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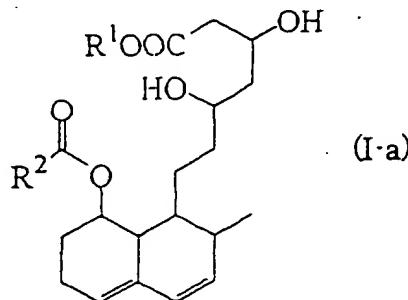
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(54) PROCESS FOR PRODUCING HMG-CoA REDUCTASE INHIBITOR

(57) The present invention relates to a protein derived from a microorganism belonging to the genus *Bacillus*, which has an activity of hydroxylating a compound represented by the formula (I-a):

wherein R¹ represents a hydrogen atom, a substituted or unsubstituted alkyl, or an alkali metal, and R² represents a substituted or unsubstituted alkyl or a substituted or unsubstituted aryl,
or a ring-closed lactone form thereof;
a DNA encoding said protein; and a recombinant DNA comprising said DNA.



(I-a)

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DescriptionTechnical Field

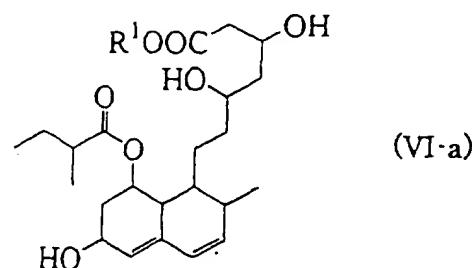
5 [0001] The present invention relates to a DNA which is related to the production of a compound which inhibits hydroxymethylglutaryl CoA(HMG-CoA) reductase and has an action of reducing serum cholesterol, and a process for producing said compound using the DNA.

Background Art

10 [0002] A compound represented by the formula (VI-a) (hereinafter referred to as compound (VI-a)):

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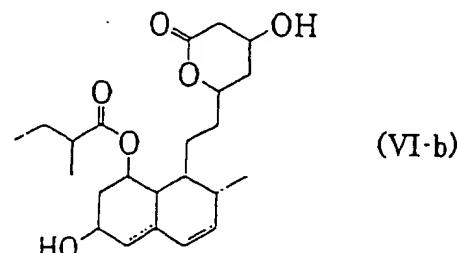
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25 wherein R¹ represents a hydrogen atom, a substituted or unsubstituted alkyl, or an alkali metal; or a lactone form of compound (VI-a) represented by the formula (VI-b) (hereinafter referred to as compound (VI-b)):

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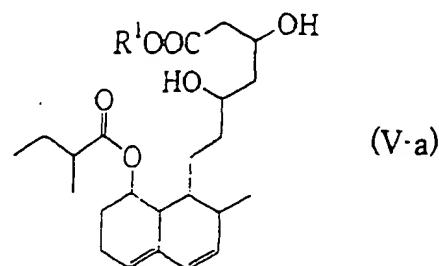
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is known to inhibit HMG-CoA reductase and exhibit an action of reducing serum cholesterol (*The Journal of Antibiotics*, 29, 1346 (1976)).

45 [0003] There have been several reports regarding methods for producing compound (VI-a) or compound (VI-b) from a compound represented by the formula (V-a) (hereinafter referred to as compound (V-a)):

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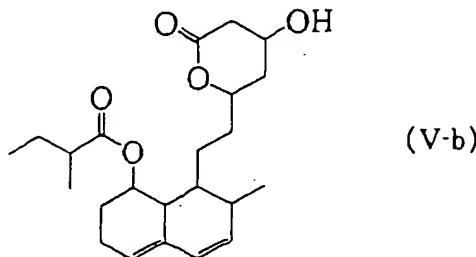


wherein R¹ has the same definition as the above; or from the lactone form of compound (V-a) represented by the

formula (V-b) (hereinafter referred to as compound (V-b):

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15 using a microorganism.

[0004] Specifically, Japanese Patent Application Laid-Open (kokai) No. 57-50894 describes a method which uses filamentous fungi; both Japanese Patent Application Laid-Open (kokai) No. 7-184670 and International Publication WO96/40863 describe a method which uses *Actinomycetes*; and Japanese Patent No. 2672551 describes a method which uses recombinant *Actinomycetes*. As is well known, however, since filamentous fungi and *Actinomycetes* grow with filamentous form by elongating hyphae, the viscosity of the culture in a fermentor increases.

[0005] This often causes a shortage of oxygen in the culture, and since the culture becomes heterogeneous, reaction efficiency tends to be reduced. In order to resolve this oxygen shortage and maintain homogenousness of the culture, the agitation rate of the fermentor should be raised, but by raising the agitation rate, hyphae are sheared and, as a result, activity of the microorganisms tends to decrease (Basic Fermentation Engineering (Hakko Kogaku no Kiso) p. 169-190, P.F. Stansbury, A. Whitaker, Japan Scientific Societies Press (1988)).

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Disclosure of the Invention

[0006] The object of the present invention is to provide a DNA encoding a novel, hydroxylase, and an industrially advantageous method for producing a compound which inhibits HMG-CoA reductase and has an action of reducing the level of serum cholesterol.

[0007] The present inventors considered that, if the hydroxylation of compound (I-a) or compound (I-b) could be carried out with a microorganism forming no hyphae, inconvenience such as the decrease of reaction efficiency due to the heterogeneity of the culture caused by hyphae formation could be avoided, and that this would be industrially advantageous. Thus, as a result of intensive studies, the present inventors have accomplished the present invention.

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[0008] Thus, the present invention relates to the following (1) to (39).

[0009] Hereinafter, in the formulas, R¹ represents a hydrogen atom, a substituted or unsubstituted alkyl, or an alkali metal, and R² represents a substituted or unsubstituted alkyl, or a substituted or unsubstituted aryl, unless otherwise specified.

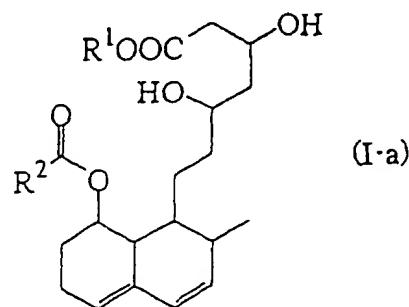
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(1) A protein which is derived from a microorganism belonging to the genus *Bacillus*, and has an activity of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b),
wherein the compound (I-a) is a compound represented by the formula (I-a):

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the compound (I-b) is a lactone form of compound (I-a) and is represented by the formula (I-b):

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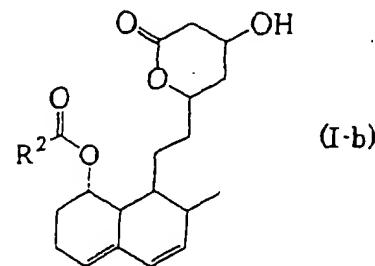
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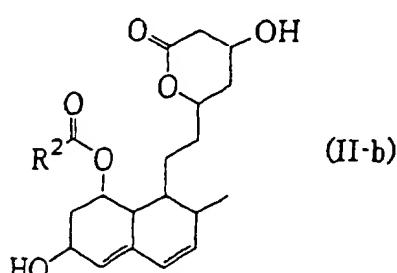
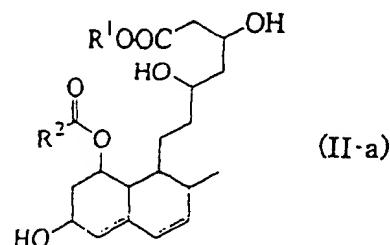
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the compound (II-a) is a compound represented by the formula (II-a):

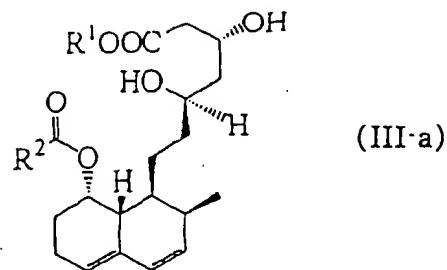


(2) A protein which is derived from a microorganism belonging to the genus *Bacillus*, and has an activity of producing compound (IV-a) or compound (IV-b) from compound (III-a) or compound (III-b), wherein the compound (III-a) is a compound represented by the formula (III-a):

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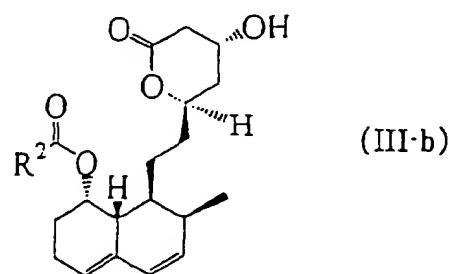


the compound (III-b) is a lactone form of compound (III-a) and is represented by the formula (III-b):

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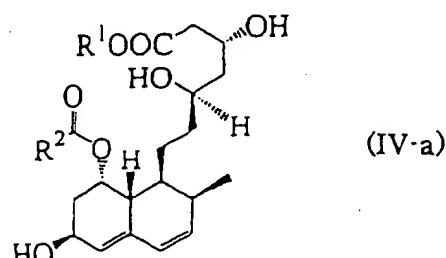


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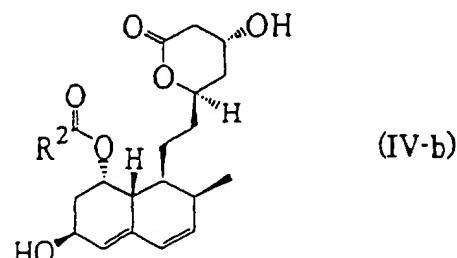


the compound (IV-b) is a lactone form of compound (IV-a) and is represented by the formula (IV-b):

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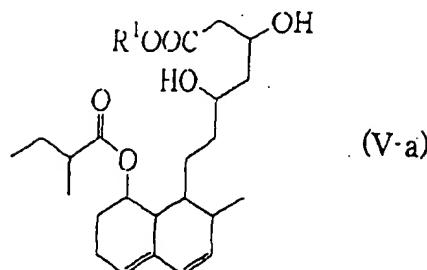
(3) A protein which is derived from a microorganism belonging to the genus *Bacillus*, and has an activity of producing compound (VI-a) or compound (VI-b) from compound (V-a) or compound (V-b),

wherein the compound (V-a) is a compound represented by the formula (V-a):

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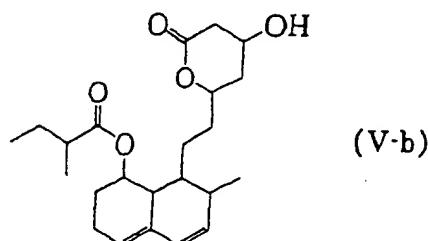
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(V-a)

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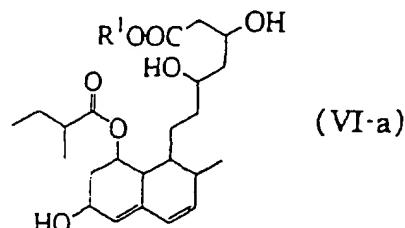
(V-b)

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the compound (VI-a) is a compound represented by the formula (VI-a):

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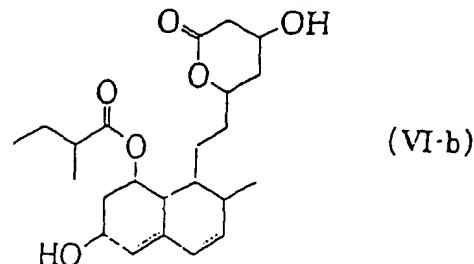
(VI-a)

; and

45 the compound (VI-b) is a lactone form of compound (VI-a) and is represented by the formula (VI-b):

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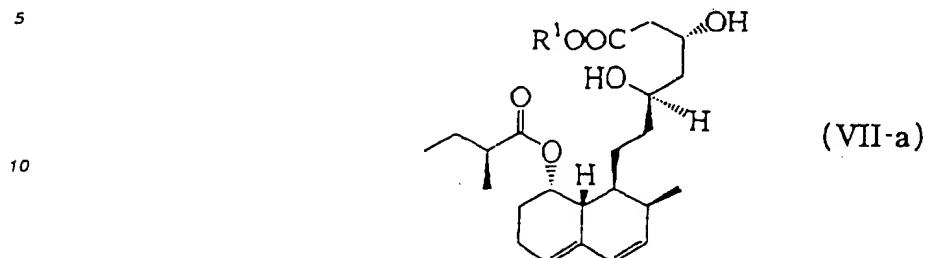
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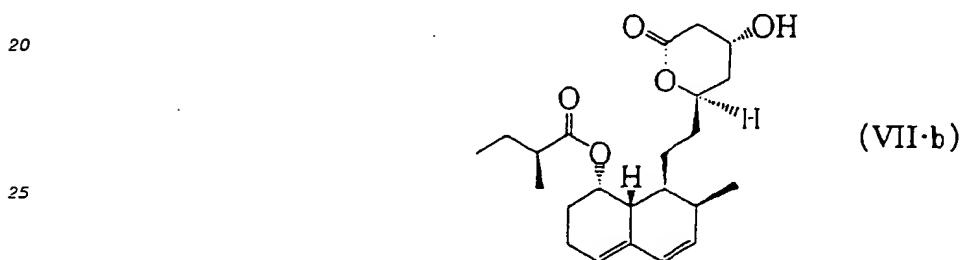
(VI-b)

(4) A protein which is derived from a microorganism belonging to the genus *Bacillus*, and has an activity of producing

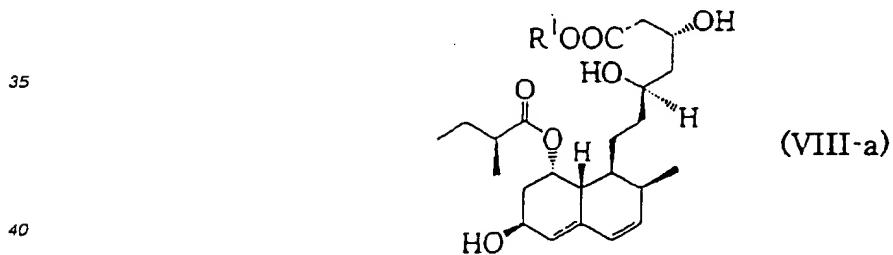
compound (VIII-a) or compound (VIII-b) from compound (VII-a) or compound (VII-b), wherein the compound (VII-a) is a compound represented by the formula (VII-a):



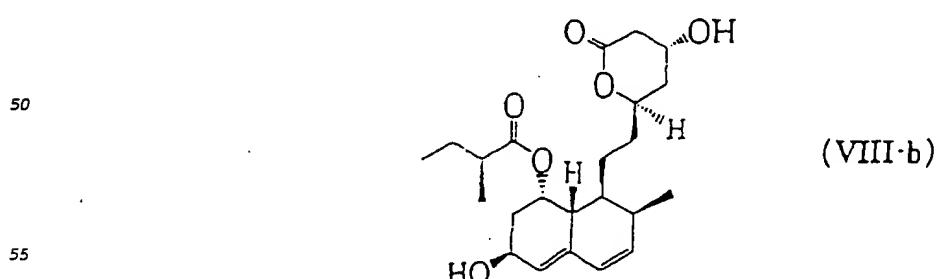
the compound (VII-b) is a lactone form of compound (VII-a) and is represented by the formula (VII-b):



30 the compound (VIII-a) is a compound represented by the formula (VIII-a):



; and the compound (VIII-b) is a lactone form of compound (VIII-a) and is represented by the formula (VIII-b):



(5) The protein according to any one of (1) to (4) above, wherein the microorganism belonging to the genus *Bacillus* is a microorganism selected from *B. subtilis*, *B. megaterium*, *B. laterosporus*, *B. sphaericus*, *B. pumilus*, *B. stearothermophilus*, *B. cereus*, *B. badius*, *B. brevis*, *B. alvei*, *B. circulans* and *B. macerans*.

5 (6) The protein according to any one of (1) to (5) above, wherein the microorganism belonging to the genus *Bacillus* is a microorganism selected from *B. subtilis* ATCC6051, *B. megaterium* ATCC10778, *B. megaterium* ATCC11562, *B. megaterium* ATCC13402, *B. megaterium* ATCC15177, *B. megaterium* ATCC15450, *B. megaterium* ATCC19213, *B. megaterium* IAM1032, *B. laterosporus* ATCC4517, *B. pumilus* FERM BP-2064, *B. badius* ATCC14574, *B. brevis* NRRL B-8029, *B. alvei* ATCC6344, *B. circulans* NTCT-2610, and *B. macerans* NCIMB-9368.

10 (7) The protein according to any one of (1) to (5) above, wherein the microorganism belonging to the genus *Bacillus* is a microorganism selected from *Bacillus* sp. FERM BP-6029 or *Bacillus* sp. FERM BP-6030.

15 (8) A protein having the amino acid sequence shown by SEQ ID NO: 1.

(9) A protein which has an amino acid sequence comprising deletion, substitution or addition of one or more amino acids in the amino acid sequence shown by SEQ ID NO: 1, and has an activity of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b).

20 (10) The protein according to (9) above, wherein the protein has the amino acid sequence shown by SEQ ID NO: 42 or 45.

(11) The protein according to (9) above, wherein the compound (I-a) is compound (III-a), the compound (I-b) is compound (III-b), the compound (II-a) is compound (IV-a), and the compound (II-b) is compound (IV-b).

25 (12) The protein according to (9) above, wherein the compound (I-a) is compound (V-a), the compound (I-b) is compound (V-b), the compound (II-a) is compound (VI-a), and the compound (II-b) is compound (VI-b).

(13) The protein according to (9) above, wherein the compound (I-a) is compound (VII-a), the compound (I-b) is compound (VII-b), the compound (II-a) is compound (VIII-a), and the compound (II-b) is compound (VIII-b).

30 (14) An isolated DNA having the nucleotide sequence shown by SEQ ID NO: 2.

(15) An isolated DNA which hybridizes with the DNA according to (14) above under stringent conditions, and encodes a protein having an activity of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b).

(16) The DNA according to (15) above, wherein the DNA has a nucleotide sequence selected from the group consisting of the nucleotide sequences shown by SEQ ID NOS: 41, 43 and 44.

40 (17) An isolated DNA encoding the protein according to any one of (1) to (12) above.

(18) The DNA according to (15) above, wherein the compound (I-a) is compound (III-a), the compound (I-b) is compound (III-b), the compound (II-a) is compound (IV-a), and the compound (II-b) is compound (IV-b).

45 (19) The DNA according to (15) above, wherein the compound (I-a) is compound (V-a), the compound (I-b) is compound (V-b), the compound (II-a) is compound (VI-a), and the compound (II-b) is compound (VI-b).

(20) The DNA according to (15) above, wherein the compound (I-a) is compound (VII-a), the compound (I-b) is compound (VII-b), the compound (II-a) is compound (VIII-a), and the compound (II-b) is compound (VIII-b).

50 (21) A recombinant DNA vector comprising the DNA according to any one of (14) to (20) above.

(22) A transformant obtained by introducing the recombinant DNA vector according to (21) above into a host cell.

55 (23) The transformant according to (22) above, wherein the transformant belongs to a microorganism selected from the genera *Escherichia*, *Bacillus*, *Corynebacterium*, and *Streptomyces*.

(24) The transformant according to (22) or (23) above, wherein the transformant belongs to microorganism selected from *Escherichia coli*, *Bacillus subtilis*, *Bacillus megaterium*, *Corynebacterium glutamicum*, *Corynebacterium ammoniagenes*, *Corynebacterium callunae* and *Streptomyces lividans*.

5 (25) A process for producing compound (II-a) or compound (II-b), wherein the transformant according to any one of (22) to (24) above, a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:

10 allowing compound (I-a) or compound (I-b) to exist in an aqueous medium;
allowing compound (II-a) or compound (II-b) to be produced and accumulated in said aqueous medium; and
collecting compound (II-a) or compound (II-b) from said aqueous medium.

15 (26) A process for producing compound (IV-a) or compound (IV-b), wherein the transformant according to any one of (22) to (24) above, a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:

20 allowing compound (III-a) or compound (III-b) to exist in an aqueous medium;
allowing compound (IV-a) or compound (IV-b) to be produced and accumulated in said aqueous medium; and
collecting compound (IV-a) or compound (IV-b) from said aqueous medium.

25 (27) A process for producing compound (VI-a) or compound (VI-b), wherein the transformant according to any one of (22) to (24) above, a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:

30 allowing compound (V-a) or compound (V-b) to exist in an aqueous medium;
allowing compound (VI-a) or compound (VI-b) to be produced and accumulated in said aqueous medium; and
collecting compound (VI-a) or compound (VI-b) from said aqueous medium.

35 (28) A process for producing compound (VIII-a) or compound (VIII-b), wherein the transformant according to any one of (22) to (24) above, a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:

40 allowing compound (VII-a) or compound (VII-b) to exist in an aqueous medium;
allowing compound (VIII-a) or compound (VIII-b) to be produced and accumulated in said aqueous medium;
and
collecting compound (VIII-a) or compound (VIII-b) from said aqueous medium.

45 (29) The process according to (25) above, wherein the compound (II-b) is the compound (II-b) obtained by forming a lacton from compound (II-a).

50 (30) The process according to (25) above, wherein the compound (II-a) is the compound (II-a) obtained by opening the lactone ring of compound (II-b).

55 (31) The process according to (26) above, wherein the compound (IV-b) is the compound (IV-b) obtained by forming a lacton from compound (IV-a).

(32) The process according to (26) above, wherein the compound (IV-a) is the compound (IV-a) obtained by opening the lactone ring of compound (IV-b).

(33) The process according to (27) above, wherein the compound (VI-b) is the compound (VI-b) obtained by forming a lacton from compound (VI-a).

(34) The process according to (27) above, wherein the compound (VI-a) is the compound (VI-a) obtained by opening the lactone ring of compound (VI-b).

55 (35) The process according to (28) above, wherein the compound (VIII-b) is the compound (VIII-b) obtained by forming a lacton from compound (VIII-a).

(36) The process according to (28) above, wherein the compound (VIII-a) is the compound (VIII-a) obtained by opening the lactone ring of compound (VIII-b).

5 (37) The process according to any one of (25) to (28) above, wherein the treated product of the culture of the transformant is a treated product selected from cultured cells; treated products such as dried cells, freeze-dried cells, cells treated with a surfactant, cells treated with an enzyme, cells treated by ultrasonication, cells treated by mechanical milling, cells treated by solvent; a protein fraction of a cell; and an immobilized products of cells or treated cells.

10 (38) A process for producing a protein, which comprises culturing the transformant according to any one of (22) to (24) above in a medium; producing and accumulating the protein according to any one of (1) to (12) above in the culture; and collecting said protein from said culture.

15 (39) An oligonucleotide corresponding to a sequence consisting of 5 to 60 continuous nucleotides in a nucleotide sequence selected from the group consisting of the nucleotide sequences shown by SEQ ID NOS: 2, 41, 43 and 44; or an oligonucleotide corresponding to a complementary sequence to said oligonucleotide.

[0010] The present invention will be described in detail below.

20 1. Obtaining of yjiB gene

[0011] The DNA of the present invention can be obtained by PCR method [Science. 230, 1350 (1985)] using the genome nucleotide sequence information of a chromosome of *Bacillus subtilis* which has already been determined [http://www.pasteur.fr/Bio/SubtiList.html] and the information on *Bacillus subtilis* yjiB gene deduced from said genome nucleotide sequence.

25 [0012] Specifically, the DNA of the present invention can be obtained by the following method.

[0013] *Bacillus subtilis* (e.g., *B. subtilis* ATCC15563) is cultured by a usual manner in a medium suitable for *Bacillus subtilis*, e.g. LB liquid medium [containing Bacto Trypton (produced by Difco) 10g, yeast extract (produced by Difco) 5g, and NaCl 5g in 1L of water; and adjusted to pH 7.2]. After culturing, the cells are collected from the culture by centrifugation.

30 [0014] A chromosomal DNA is isolated from the collected cells by a known method (e.g., Molecular Cloning 2nd ed).

[0015] Using the nucleotide sequence information shown by SEQ ID NO:2, sense and antisense primers containing nucleotide sequences corresponding to the DNA region encoding a protein of the present invention are synthesized with a DNA synthesizer.

35 [0016] After amplification by PCR, in order to enable introduction of said amplified DNA fragments into a plasmid, it is preferred that an appropriate restriction site such as BamHI, EcoRI or the like is added at 5' end of the sense and antisense primers.

[0017] Examples of combinations of said sense and antisense primers include combination of DNAs having nucleotide sequences shown by SEQ ID NOS:13 and 14.

40 [0018] Using chromosomal DNA as a template, PCR is performed with these primers. TaKaRa LA-PCR™ Kit Ver. 2 (TaKaRa), Expand™ High-Fidelity PCR System (Boehringer Mannheim) or the like by a DNA Thermal Cycler (Perkin-Elmer Japan).

[0019] When PCR is performed, for example, the following method can be carried out. In the case where the above primer is a DNA fragment of 2kb or less, each cycle consists of reaction steps of 30 seconds at 94°C, 30 seconds to 45 1 minute at 55°C, and 2 minutes at 72°C. In the case where the above primer is a DNA fragment of more than 2kb, each cycle consists of reaction steps of 20 seconds at 98°C and 3 minutes at 68°C. In any case, PCR is performed under conditions where the 30 cycles are repeated, and then reaction is carried out for 7 minutes at 72°C.

[0020] The amplified DNA fragments are cut at the same restriction site as the site which is formed using the above primers, and then the DNA fragments are fractioned and recovered by a method such as agarose gel electrophoresis, sucrose density gradient ultracentrifugation and the like.

50 [0021] Using the recovered DNA fragments, a cloning vector is produced by a usual method such as methods described in Molecular Cloning 2nd ed., Current Protocols in Molecular Biology, Supplement 1-38, John Wiley & Sons (1987-1997) (abbreviated as Current Protocols in Molecular Biology, Supplement hereinafter), DNA Cloning 1: Core Techniques. A Practical Approach. Second Edition, Oxford University Press (1995), or by using a commercially available kit such as Superscript Plasmid System for cDNA Synthesis and Plasmid Cloning (produced by Life Technologies), ZAP-cDNA Synthesis Kit (produced by Stratagene), etc., then the thus-produced cloning vector is used to transform *Escherichia coli*, e.g. *E.coli* DH5 α strain (available from TOYOBO).

[0022] Examples of a cloning vector for the transformation of *E. coli* include a phage vector and plasmid vector

insofar as it is capable of self-replicating in *E. coli* K12 strain. An expression vector for *E. coli* can also be used as a cloning vector. Specifically, examples thereof include ZAP Express [produced by Stratagene. Strategies, 5, 58 (1992)], pBluescript II SK(+) [Nucleic Acids Research, 17, 9494 (1989)], Lambda ZAP II (produced by Stratagene), λ gt10, λ gt11 [DNA Cloning. A Practical Approach, 1, 49 (1985)], λ TriplEx (produced by Clonetech), λ ExCell (produced by Pharmacia), pT7T318U (produced by Pharmacia), pCD2 [H. Okayama and P. Berg : Mol. Cell. Biol., 3, 280 (1983)], pMW218 (produced by Wako Pure Chemical Industries), pUC118, pSTV28 (produced by Takara), pEG400 [J. Bac., 172, 2392 (1990)], pHMV1520 (produced by MoBiTec), pOE-30 (produced by QIAGEN), etc.

5 [0023] A plasmid containing a desired DNA can be obtained from the obtained transformed strain by usual methods described in e.g. Molecular Cloning 2nd edition, Current Protocols in Molecular Biology Supplement, DNA Cloning 1: Core Techniques. A Practical Approach, Second Edition, and Oxford University Press (1995), etc.

10 [0024] Using the aforementioned method, a plasmid containing a DNA encoding a protein which catalyzes reaction of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b), can be obtained.

[0025] Examples of the plasmids include the below-mentioned pSyjIB.

15 [0026] Apart from the aforementioned method, a plasmid containing a DNA encoding a protein which catalyzes a reaction of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b) can be obtained also by a method wherein a chromosomal library of *Bacillus subtilis* is prepared with a suitable vector using *E. coli* as a host, and the activity of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b) is measured on each strain of this library.

20 [0027] The nucleotide sequence of the above-obtained gene can be used to obtain homologues of the DNA from other prokaryotes or plants in the same manner as mentioned above.

[0028] The DNA and DNA fragment of the present invention obtained in the above method can be used to prepare oligonucleotides such as antisense oligonucleotides, sense oligonucleotides etc. having a partial sequence of the DNA of the present invention or such oligonucleotides containing RNAs. Alternatively, based on the sequence information of the above-obtained DNA, these oligonucleotides can be synthesized with the above DNA synthesizer.

25 [0029] Examples of the oligonucleotides include a DNA having the same sequence as a contiguous 5 to 60 nucleotides in the nucleotide sequence of the above DNA, or a DNA having a complementary sequence to said DNA. RNAs having complementary sequences to these DNAs are also oligonucleotides of the present invention.

[0030] Examples of said oligonucleotides include a DNA having the same sequence as a contiguous 5 to 60 nucleotides sequence in the nucleotide sequences shown by SEQ ID NOS: 2, 41, 43 or 44, or a DNA having a complementary sequence to said DNA. If these are used as sense and antisense primers, the aforementioned oligonucleotides without extreme difference in melting temperatures (T_m) and numbers of bases are preferably used. Specifically, examples thereof include oligonucleotides having a nucleotide sequence shown by SEQ ID NOS: 3 to 39.

30 [0031] Furthermore, derivatives of these oligonucleotides (referred to as oligonucleotide derivative hereinafter) can also be used as the DNA of the present invention.

[0032] Oligonucleotide derivatives include a oligonucleotide derivative whose phosphate diester linkage is replaced by a phosphorothioate linkage, an oligonucleotide derivative whose phosphate diester linkage is replaced by a N3'-P5' phosphoamidate linkage, an oligonucleotide derivative whose ribose and phosphate diester linkage is replaced by a peptide-nucleic acid linkage, an oligonucleotide derivative whose uracil is replaced by C-5 propinyl uracil, an oligonucleotide derivative whose uracil is replaced by C-5 thiazol uracil, an oligonucleotide derivative whose cytosine is replaced by C-5 propinyl cytosine, an oligonucleotide derivative whose cytosine is replaced by phenoxazine-modified cytosine, an oligonucleotide derivative whose ribose is replaced by 2'-O-propyl ribose, or an oligonucleotide derivative whose ribose is replaced by 2'-methoxy-ethoxyribose, etc. [Saibo Kogaku, 16, 1463 (1997).]

35 II. Method for producing a protein which catalyzes a reaction of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b)

[0033] In order to express the above-obtained DNA in a host cell, the desired DNA fragment is cut into a fragment of suitable length containing said gene using restriction enzymes or DNase enzymes, followed by inserting the fragment into a site downstream of a promoter in an expression vector, and then the expression vector is introduced into host cells suitable for use of the expression vector.

40 [0034] The host cells may be any of bacteria, yeasts, animal cells, insect cells or the like insofar as they can express the objective gene.

[0035] As an expression vector, a vector capable of being autonomously replicated in a host cell or capable of being integrated into a chromosome, and containing a promoter at a site suitable for transcription of the above objective gene, is used.

45 [0036] When prokaryotes such as bacteria are used as the host cell, the expression vector for expressing the above DNA is preferably a vector autonomously replicable in said cell and is a recombinant vector composed of a promoter, a ribosome-binding sequence, the above DNA and a transcription termination sequence. A gene for regulating the

promoter may be contained.

[0037] The expression vectors include pBTrp2, pBTac1, pBTac2 (all of which are commercially available from Boehringer Mannheim), pKK233-2 (produced by Pharmacia), pSE280 (produced by Invitrogen), pGEMEX-1 (produced by Promega), pQE-8 (produced by QIAGEN), pQE-30 (produced by QIAGEN), pKYP10 (Japanese Patent Application Laid-Open No. 58-110600), pKYP200 [Agricultural Biological Chemistry, 48, 669 (1984)], pLSA1 [Agric. Biol. Chem., 53, 277 (1989)], pGEL1 [Proc. Natl. Acad. Sci., USA, 82, 4306 (1985)], pBluescriptII SK(+), pBluescriptII SK(-) (produced by Stratagene), pTrS30 (FERM BP-5407), pTrS32 (FERM BP-5408), pGEX (produced by Pharmacia), pET-3 (produced by Novagen), pTerm2 (US 4,686,191, US 4,939,094, US 5,160,735), pSupex, pUB110, pTP5, pC194, pUC18 [gene, 33, 103 (1985)], pUC19 [Gene, 33, 103 (1985)], pSTV28 (produced by Takara), pSTV29 (produced by Takara), 10 pUC118 (produced by Takara), pPA1 (Japanese Patent Application Laid-Open No. 63-233798), pEG400 [J. Bacteriol., 172, 2392 (1990)], pQE-30 (produced by QIAGEN), PHY300 (produced by Takara), pHW1520 (produced by MoBiTec), etc.

[0038] The promoter may be any one insofar as it can be expressed in a host cell. Examples are promoters derived from *E.coli*, phage etc., such as trp promoter (P_{trp}), lac promoter (P_{lac}), PL promoter, PR promoter and PSE promoter, and SP01 promoter, SP02 promoter, penP promoter and the like. Artificially designed and modified promoters such as a P_{trg}>2 promoter having two P_{trp} promoters in tandem, tac promoter, lacI promoter, and lacT7 promoter can also be used. Furthermore, xylA promoter for expression in *Bacillus* bacteria or P54-6 promoter for expression in *Corynebacterium* bacteria can also be used.

[0039] Any ribosome binding sequences may be used insofar as they can work in a host cell, and a plasmid in which the distance between a Shine-Dalgarno sequence and an initiation codon is adjusted to an appropriate distance (for example, 6 to 18 bases) may be preferably used.

[0040] For efficient transcription and translation, a protein which catalyzes the reaction of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b) wherein the N-terminus or a part thereof is deleted may be fused to the N-terminus part of a protein encoded by the expression vector, and the thus-obtained fused protein may be expressed. Such examples include the below-mentioned pWYjIB.

[0041] Although a transcription termination sequence is not necessarily required for expression of the desired DNA, it is preferred to locate the transcription termination sequence just downstream from the structural gene.

[0042] Examples of prokaryotes include microorganisms belonging to the genus *Escherichia*, *Corynebacterium*, *Brevibacterium*, *Bacillus*, *Microbacterium*, *Serratia*, *Pseudomonas*, *Agrobacterium*, *Alicyclobacillus*, *Anabaena*, *Anacystis*, *Arthrobacter*, *Azotobacter*, *Chromatium*, *Erwinia*, *Methylobacterium*, *Phormidium*, *Rhodobacter*, *Rhodopseudomonas*, *Rhodospirillum*, *Streptomyces*, *Synechococcus*, and *Zymomonas*, preferably *Escherichia*, *Corynebacterium*, *Brevibacterium*, *Bacillus*, *Pseudomonas*, *Agrobacterium*, *Alicyclobacillus*, *Anabaena*, *Anacystis*, *Arthrobacter*, *Azotobacter*, *Chromatium*, *Erwinia*, *Methylobacterium*, *Phormidium*, *Rhodobacter*, *Rhodopseudomonas*, *Rhodospirillum*, *Streptomyces*, *Synechococcus*, and *Zymomonas*.

[0043] Specific examples of the microorganisms include *Escherichia coli* XL1-Blue, *Escherichia coli* XL2-Blue, *Escherichia coli* DH1, *Escherichia coli* DH5 α , *Escherichia coli* MC1000, *Escherichia coli* KY3276, *Escherichia coli* W1485, *Escherichia coli* JM109, *Escherichia coli* HB101, *Escherichia coli* No.49, *Escherichia coli* W3110, *Escherichia coli* NY49, *Escherichia coli* MP347, *Escherichia coli* NM522, *Bacillus subtilis* ATCC33712, *Bacillus megaterium*, *Bacillus* sp. FERM BP-6030, *Bacillus amyloliquefaciens*, *Brevibacterium ammoniagenes*, *Brevibacterium immariophilum* ATCC14068, *Brevibacterium saccharolyticum* ATCC14066, *Brevibacterium flavum* ATCC14067, *Brevibacterium lactofermentum* ATCC13869, *Corynebacterium glutamicum* ATCC13032, *Corynebacterium glutamicum* ATCC14297, *Corynebacterium acetoacidophilum* ATCC13870, *Corynebacterium calluna* ATCC15991, *Microbacterium ammoniaphilum* ATCC15354, *Serratia ficaria*, *Serratia fonticola*, *Serratia liquefaciens*, *Serratia marcescens*, *Pseudomonas* sp. D-0110, *Agrobacterium radiobacter*, *Agrobacterium rhizogenes*, *Agrobacterium rubi*, *Anabaena cylindrical*, *Anabaena doliolum*, *Anabaena flos-aquae*, *Arthrobacter aurescens*, *Arthrobacter citreus*, *Arthrobacter globiformis*, *Arthrobacter hydrocarboglutamicus*, *Arthrobacter mysorens*, *Arthrobacter nicotianae*, *Arthrobacter parafineus*, *Arthrobacter protoformiae*, *Arthrobacter roseoparaflinus*, *Arthrobacter sulfurous*, *Arthrobacter ureafaciens*, *Chromatium buderii*, *Chromatium tepidum*, *Chromatium vinosum*, *Chromatium warmingii*, *Chromatium fluviatile*, *Erwinia uredovora*, *Erwinia carotovora*, *Erwinia ananas*, *Erwinia herbicola*, *Erwinia punctata*, *Erwinia terreus*, *Methylobacterium rhodesianum*, *Methylobacterium extorquens*, *Phormidium* sp. ATCC29409, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Rhodopseudomonas* *blastica*, *Rhodopseudomonas marina*, *Rhodopseudomonas palustris*, *Rhodospirillum rubrum*, *Rhodospirillum salexigens*, *Rhodospirillum salinarum*, *Streptomyces ambolaciens*, *Streptomyces aureofaciens*, *Streptomyces aureus*, *Streptomyces lungicidicus*, *Streptomyces griseochromogenes*, *Streptomyces griseus*, *Streptomyces lividans*, *Streptomyces olivogriseus*, *Streptomyces rameus*, *Streptomyces tanashiensis*, *Streptomyces vinaceus*, and *Zymomonas mobilis*.

[0044] The method for introducing the recombinant vector may be any method for introducing DNA into the host cells described above. For examples, mention can be made of a method using calcium ions [Proc. Natl. Acad. Sci. USA, 69, 2110 (1972)], a protoplast method (Japanese Patent Application Laid-Open No. 63-248394), an electroporation

method, a method described in Gene, 17, 107 (1982) and Molecular & General Genetics, 168, 111 (1979), and the like. [0045] If yeasts are used as the host cell, expression vectors such as YEp13 (ATCC37115), YEp24 (ATCC37051), YCp50 (ATCC37419), pHS19, and pHS15 can be exemplified.

[0046] Any promoter can be used insofar as they can be expressed in yeasts. For example, mention can be made of promoters such as PHO5 promoter, PGK promoter, GAP promoter, ADH promoter, gal 1 promoter, gal 10 promoter, heat shock protein promoter, MF α 1 promoter, and CUP 1 promoter.

[0047] Examples of host cells include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Trichosporon pullulans*, and *Schwanniomyces alluvius*.

[0048] The method for introducing a recombinant vector may be any method for introducing DNA into yeast, and examples include an electroporation method [Methods Enzymol., 194, 182 (1990)], a spheroplast method [Proc. Natl. Acad. Sci. USA, 75, 1929 (1978)], a lithium acetate method [J. Bacteriol., 153, 163 (1983)] and a method described in Proc. Natl. Acad. Sci. USA, 75, 1929 (1978).

[0049] If animal cells are used as the host cells, expression vectors such as pcDNA1, pcDM8 (commercially available from Funakoshi), pAGE107 (Japanese Patent Application Laid-Open No. 3-22979; Cytotechnology, 3, 133 (1990)), pAS3-3 (Japanese Patent Application Laid-Open No. 2-227075), pCDM8 [Nature, 329, 840 (1987)], pcDNA1/Amp (Invitrogen), pREP4 (Invitrogen), pAGE103 [J. Biochem., 101, 1307 (1987)], and pAGE210 can be used.

[0050] The promoter to be used may be any promoter which can be expressed in animal cells. Examples are a promoter for IE (immediate early) gene of cytomegalovirus (human CMV), SV40 early promoter, retrovirus promoter, metallothionein promoter, heat shock promoter, Sr α promoter and the like. Furthermore, an enhancer of the IE gene of human CMV may be used together with a promoter.

[0051] Examples of animal cells include Namalwa cell, HBT5637 (Japanese Patent Application Laid-Open No. 63-299), COS1 cell, COS7 cell, CHO cell and the like.

[0052] The method for introducing a recombinant vector into animal cells may be any method for introducing DNA into animal cells. Examples of such methods include an electroporation method [Cytotechnology, 3, 133 (1990)], a calcium phosphate method (Japanese Patent Application Laid-Open No. 2-227075), a lipofection method [Proc. Natl. Acad. Sci., USA, 84, 7413 (1987)], a method described in Virology, 52, 456 (1973), and the like. Obtaining and culturing of the transformant can be conducted according to methods described in Japanese Patent Application Laid-Open No. 2-227075 or Japanese Patent Application Laid-Open No. 2-257891.

[0053] If insect cells are used as the host cells, the protein can be expressed by methods described in *Baculovirus Expression Vectors*, A Laboratory Manual, Current Protocols in Molecular Biology Supplement 1-38 (1987-1997); *Bio/Technology*, 6, 47 (1988) and the like.

[0054] That is, a recombinant gene transfer vector and a baculovirus are co-transfected into insect cells to obtain a recombinant virus in the culture supernatant of the insect cells, and then the insect cells are infected with the recombinant virus whereby the protein can be expressed.

[0055] Examples of the gene transfer vectors used in this method include pVL1392, pVL1393 and pBlueBacIII (all manufactured by Invitrogen).

[0056] As the baculovirus, it is possible to employ, e.g. *Autographa californica* nuclear polyhedrosis virus, that is, a virus infecting insects of the family Barathra.

[0057] As the insect cells, it is possible to use Sf9, Sf21 [*Baculovirus Expression Vectors*, A Laboratory Manual, W. H. Freeman and Company, New York (1992)] which are oocytes of *Spodoptera frugiperda* and High 5 (Invitrogen) which is oocyte of *Trichoplusia ni*, and the like.

[0058] As a method for co-transferring the aforesaid recombinant gene transfer vector and the aforesaid baculovirus into insect cells for preparing the recombinant virus, for example, a calcium phosphate method (Japanese Patent Application Laid-Open No. 2-227075), a lipofection method [Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)] and the like may be used.

[0059] As a method for expressing gene, in addition to direct expression, secretory production, expression of a fusion protein and the like can be carried out according to the method described in *Molecular Cloning* 2nd edition.

[0060] When a protein has been expressed by yeasts, animal cells or insect cells, the protein to which a sugar or sugar chain is added can be obtained.

[0061] The thus-obtained transformant is cultured in a medium to produce and accumulate proteins which catalyze the reaction of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b) in the culture, and the proteins are recovered from the culture, thereby producing the protein which catalyzes production of compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b).

[0062] As a method for culturing in a medium the transformant for the production of the protein of the present invention which catalyzes the reaction of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b), conventional methods used for culturing a transformant in a host cell can be used.

[0063] If the transformant of the present invention is a prokaryote such as *E.coli* or an eukaryote such as yeast, the medium for culturing these organisms may be either a natural or synthetic medium insofar as it contains a carbon

source, a nitrogen source, inorganic salts and the like which can be assimilated by said organisms, and it allows efficient culturing of the transformant.

[0064] As a carbon source, any carbon source can be used as long as it can be assimilated by the microorganisms, including carbohydrates such as glucose, fructose, sucrose, or molasses containing those sources, starch or starch hydrolysates; organic acids such as acetic acid, propionic acid; and alcohols such as ethanol, propanol.

[0065] As a nitrogen source, the following can be used: ammonia; ammonium salts of various inorganic acids and organic acids, such as ammonium chloride, ammonium sulfate, ammonium acetate, and ammonium phosphate; other nitrogen-containing compounds; and peptone, meat extracts, yeast extracts, corn steep liquor, casein hydrolysates, soy bean meal, soy bean meal hydrolysates, various fermented cells and hydrolysates thereof, and the like.

[0066] Examples of the inorganic substances include potassium dihydrogenphosphate, potassium hydrogenphosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate and calcium carbonate.

[0067] The culturing is carried out under aerobic conditions by shake culturing or aeration-agitation culturing or the like. The culturing temperature is preferably 15 to 50°C, and the culturing period is usually 16 hours to 7 days. While culturing, pH is maintained at 3.0 to 9.0. The pH control is conducted using an inorganic or organic acid, alkaline solution, urea, calcium carbonate, ammonia and the like.

[0068] If necessary, antibiotics such as ampicillin and tetracycline may be added to the medium while culturing.

[0069] When a microorganism transformed with an expression vector using an inductive promoter as a promoter is cultured, an inducer may be optionally added to the medium. For example, isopropyl- β -D-thiogalactopyranoside (IPTG), indole acrylic acid (IAA) or xylose may be added to the medium respectively, when a microorganism transformed with expression vectors containing lac promoter, trp promoter, or xylA promoter is used.

[0070] The medium for culturing the transformant obtained by using animal cells as host cells may be a generally-used medium such as RPMI1640 medium [The Journal of the American Medical Association, 199, 519 (1967)], Eagle's MEM medium [Science, 122, 501 (1952)], DMEM medium [Virology, 8, 396 (1959)], 199 medium [Proceeding of the Society for the Biological Medicine, 73, 1 (1950)] or any one of these media further supplemented with fetal calf serum.

[0071] Culturing is usually carried out for 1 to 7 days at pH 6 to 8 at 30 to 40°C in the presence of 5% CO₂.

[0072] If necessary, antibiotics such as kanamycin and penicillin may be added to the medium while culturing.

[0073] The medium for culturing the transformant obtained by using insect cells as host cells may be a generally-used medium such as TNM-FH medium (produced by Pharmingen), Sf-900 II SFM medium (produced by Gibco BRL), ExCell 400 and ExCell 405 [both are products of JRH Biosciences], Grace's Insect Medium [Grace, T.C.C., Nature, 195, 788 (1962)] or the like.

[0074] Culturing is usually carried out at pH 6 to 7 at a temperature of 25 to 30°C for a period of to 5 days.

[0075] If necessary, antibiotics such as gentamycin may be added to the medium while culturing.

[0076] For isolating and purifying the protein which catalyzes a reaction of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b) from the culture of the transformant of the present invention, any conventional methods for the isolation and purification of enzymes can be performed.

[0077] For example, in the case where the protein of the present invention is expressed in a soluble form in cells, after culturing, the cells are recovered by centrifugation and suspended in an aqueous buffer, followed by disruption with ultrasonic disrupter, French Press, Manton-Gaulin homogenizer, Dymomill or the like, thereby obtaining a cell-free extract. From the supernatant obtained by centrifuging the cell-free extract, a purified preparation can be obtained by using conventional methods for isolation and purification of enzymes alone or in combination, such as solvent extraction, salting-out or desalting with sulfate ammonium etc., precipitation with an organic solvent, anion-exchange chromatography on resin such as diethylaminoethyl(DEAE)-Sepharose, DIAION HPA-75 (produced by Mitsubishi Chemical Industries Ltd.) or the like; cation-exchange chromatography on resin such as S-Sepharose FF (Pharmacia) or the like, hydrophobic chromatography on resin such as butyl Sepharose, phenyl Sepharose or the like, gel filtration using molecular sieve, affinity chromatography, chromatofocusing, and electrophoresis such as isoelectric electrophoresis.

[0078] In the case where the protein is expressed in a form of an inclusion body in cells, the cells are similarly recovered, disrupted and centrifuged, thereby obtaining a precipitated fraction, and the protein is recovered from the fraction in a usual manner. The recovered inclusion body is solubilized with a protein denaturating agent. The solubilized solution is then diluted with or dialyzed against a solution not containing the protein denaturating agent or a solution containing the protein denaturating agent at a concentration low enough not to denature the protein, whereby the protein is renatured to have normal tertiary structure, and its purified preparation can be obtained by the same isolation and purification method as described above.

[0079] When the protein of the present invention or a saccharide modified derivatives thereof are extracellularly secreted, the protein or the derivatives to which saccharide chain is added, can be recovered from the supernatant of the culture. That is, the culture is subjected to an above-mentioned process such as centrifugation and the like, thereby obtaining soluble fractions, then a purified preparation can be obtained from said soluble fractions in the same manner as in the above.

[0080] Examples of the thus-obtained proteins include proteins having amino acid sequences shown by SEQ ID NOS: 1, 42 or 45. Furthermore, the protein expressed in the above manner can also be produced by chemically synthesis methods such as Fmoc method (fluorenyl methyloxycarbonyl method) and tBoc method (t-butyloxycarbonyl method). Alternatively, the protein can be obtained by synthesis using a peptide synthesizer manufactured by Sowa 5 Trading (Advanced chemTech, USA), Perkin-Elmer Japan (Perkin Elmer, USA), Pharmacia Biotech (Pharmacia Biotech, Sweden), ALOKA (Protein Technology Instrument, USA), KURABO (Synthecell-Vega, USA), Japan PerSeptive Ltd. (PerSeptive, USA), Shimazu, etc.

III. Production of compound (II-a) or compound (II-b)

[0081] Using cells obtained by culturing the transformant obtained in above II according to the method described in above II, a culture of said cells, a treated product of said culture, or an enzyme extracted from said cells as enzyme sources, compound (II-a) or compound (II-b) can be produced by allowing compound (I-a) or compound (I-b) to exist in an aqueous medium, allowing compound (II-a) or compound (II-b) to be produced and accumulated in the above 10 aqueous medium, and collecting compound (II-a) or compound (II-b) from the above aqueous medium.

[0082] Examples of treated products of the culture of the cells include the treated products of the cells such as dried cells, lyophilized cells, cells treated with surfactants, cells treated with enzymes, cells treated with ultrasonication, cells treated with mechanical milling, cells treated with solvents; or protein fractions of the cells; or immobilized products of 15 said cell and said treated products of said cells.

[0083] As a method for converting compound (I-a) or compound (I-b) into compound (II-a) or compound (II-b), both 20 of the following methods (a) and (b) can be used: (a) a method wherein the compound (I-a) or compound (I-b) is previously added to the medium for culturing cells; and (b) a method wherein compound (I-a) or compound (I-b) is added to the medium while culturing. Alternatively, a method wherein the enzyme source obtained from the cell culture is reacted with compound (I-a) or compound (I-b) in the aqueous medium can be also used.

[0084] In a case where compound (I-a) or compound (I-b) is added to a medium in which a microorganism is to be 25 cultured, 0.1 to 10mg, preferably 0.2 to 1mg of compound (I-a) or compound (I-b) is added to 1 ml of medium at the beginning of or at some midpoint of the culture. It is desired that compound (I-a) or compound (I-b) is added after it is dissolved in an organic solvent such as methyl alcohol or ethyl alcohol.

[0085] In a case where a method of allowing an enzyme source obtained by culturing cells to act upon compound 30 (I-a) or compound (I-b) in an aqueous medium, the amount of enzyme to be used depends on the specific activity of the enzyme source or the like. For example, when a culture of cells, cells, or a treated product thereof is used as an enzyme source, 5 to 1,000mg, preferably 10 to 400mg of enzyme source is added per 1mg of compound (I-a) or compound (I-b). The reaction is performed in an aqueous medium preferably at 20 to 50°C, and particularly preferably at 25 to 37°C. The reaction period depends on the amount, specific activity and the like of an enzyme source to be 35 used, and it is usually 2 to 150 hours, preferably 6 to 120 hours.

[0086] Examples of an aqueous medium include water, or buffers such as phosphate buffer, HEPES (N-2 hydroxyethylpiperazine-N-ethanesulfonate) buffer and Tris (tris(hydroxymethyl)aminomethane)hydrochloride buffer. An organic solvent may be added to the above buffers, unless it inhibits reaction. Examples of organic solvent include acetone, ethyl acetate, dimethyl sulfoxide, xylene, methyl alcohol, ethyl alcohol and butanol. A mixture of an organic 40 solvent and an aqueous medium is preferably used, for example when compound (I-b) is used.

[0087] In the case where compound (I-a) or compound (I-b) is added to the aqueous medium, compound (I-a) or compound (I-b) is dissolved in an aqueous medium capable of dissolving compound (I-a) or compound (I-b), and then is added to the medium. An organic solvent may be added to the above buffers, unless it inhibits reaction. Examples of organic solvents include acetone, ethyl acetate, dimethyl sulfoxide, xylene, methyl alcohol, ethyl alcohol and butanol.

[0088] Compound (I-b) and compound (II-b) can easily be converted into compound (I-a) and compound (II-a) 45 respectively by a method for opening a lactone ring as mentioned below. Likewise, compound (I-a) and compound (II-a) can easily be converted into compound (I-b) and compound (II-b) respectively by a method for producing lactone as mentioned below.

[0089] Examples of a method for opening a lactone ring include a method which comprises dissolving compound (I-b) or compound (II-b) in an aqueous medium and 50 adding thereto an acid or alkali. Examples of the aqueous medium include water and an aqueous solution containing salts, which does not inhibit the reaction, such as phosphate buffer, Tris buffer and the like. The above aqueous solution may contain an organic solvent such as methanol, ethanol, ethyl acetate and the like in a concentration which does not inhibit the reaction. Examples of acid include acetic acid, hydrochloric acid and sulfuric acid, and examples of alkali 55 include sodium hydroxide, potassium hydroxide and ammonia.

[0090] Examples of a method for producing lactone include a method which comprises dissolving compound (I-a) or compound (II-a) in a non-aqueous solvent and adding thereto an acid or base catalyst. As long as the non-aqueous solvent is an organic solvent which does not substantially contain water and can dissolve compound (I-a) or compound

(II-a). any type of non-aqueous solvent can be used. Examples of non-aqueous solvents include dichloromethane and ethyl acetate. As a catalyst, any catalyst can be used; as long as it catalyzes lactonization and does not show any actions other than lactonization on a substrate or a reaction product. Examples of the above catalyst include trifluoroacetic acid and para-toluenesulfonic acid. Reaction temperature is not particularly limited, but is preferably 0 to 100°C. and is more preferably 20 to 80°C.

[0091] The collection of compound (II-a) or compound (II-b) from the reaction solution can be carried out by any ordinary methods used in the field of organic synthetic chemistry such as extraction with organic solvents, crystallization, thin-layer chromatography, high performance liquid chromatography, and the like.

[0092] As a method for detecting and quantifying compound (II-a) or compound (II-b) obtained by the present invention, any method can be used, as long as the detection or quantification of compound (II-a) and/or compound (II-b) can be performed. Examples thereof include ^{13}C -NMR spectroscopy, ^1H -NMR spectroscopy, mass spectroscopy and high performance liquid chromatography (HPLC).

[0093] In the present invention, some compounds of compound (I-a), compound (I-b), compound (II-a) and compound (II-b) can have stereoisomers such as optical isomers. The present invention covers all possible isomers and mixtures thereof including these stereoisomers.

[0094] As compound (I-a), compound (III-a) is preferable, compound (V-a) is more preferable, and compound (VII-a) is particularly preferable.

[0095] As compound (I-b), compound (III-b) is preferable, compound (V-b) is more preferable, and compound (VII-b) is particularly preferable.

[0096] As compound (II-a), compound (IV-a) is preferable, compound (VI-a) is more preferable, and compound (VIII-a) is particularly preferable.

[0097] As compound (II-b), compound (IV-b) is preferable, compound (VI-b) is more preferable, and compound (VIII-b) is particularly preferable.

[0098] Alkyl is a linear or branched alkyl containing 1 to 10 carbon atoms, preferably 1 to 6 carbon atoms, such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, tert-butyl, pentyl, neopentyl, hexyl, isohexyl, heptyl, 4,4-dimethylpentyl, octyl, 2,2,4-trimethylpentyl, nonyl, decyl, and various branched chain isomers thereof.

[0099] Examples of aryl include phenyl and naphtyl.

[0100] The substituent in the substituted alkyl may be 1 to 3 identical or different groups, and examples thereof include halogens, hydroxy, amino, alkoxy and aryl.

[0101] The substituent in the substituted aryl may be 1 to 3 identical or different groups, and examples thereof include halogens, hydroxy, amino, alkyl and alkoxy.

[0102] The alkyl moiety in alkoxy has the same definition as in the alkyl mentioned above.

[0103] Alkali metal represents each element of lithium, sodium, potassium, rubidium, cesium or francium.

[0104] The examples of the present invention is described below, but the present invention is not limited to these examples.

BEST MODE FOR CARRYING-OUT OF THE INVENTION

Example 1: Obtaining of the DNA encoding the protein having an activity of producing compound (VIII-a) or compound (VIII-b) from compound (VII-a) or compound (VII-b)

[0105] 100mg of compound (VII-b) (produced by Sigma) was dissolved in 9.5ml of methanol, and 0.5ml of 1mol/l sodium hydroxide was added. The mixture was stirred at room temperature for 1 hour. The obtained reaction solution was dried to be solidified, and was dissolved by adding 5ml of deionized water, followed by adjusting pH to about 7 with about 0.1ml of 1mol/l hydrochloric acid. Then, 4.9ml of deionized water was added to the mixture to obtain 10ml of compound (VII-a), whose final concentration was 10mg/ml (a compound wherein, in formula (VII-a), R¹ is sodium).

[0106] *Bacillus subtilis* Marburg168 strain (ATCC15563) was inoculated with 1 platinum loop in a 10ml LB liquid medium, and cultured at 30°C overnight. After culturing, cells were collected from the obtained culture solution by centrifugation.

[0107] A chromosomal DNA was isolated and purified from the cells in a usual manner.

[0108] Sense and antisense primers having a combination of nucleotide sequences: SEQ ID NOS: 3 and 4, SEQ ID NOS: 5 and 6, SEQ ID NOS: 7 and 8, SEQ ID NOS: 9 and 10, SEQ ID NOS: 11 and 12, SEQ ID NOS: 13 and 14, and SEQ ID NOS: 15 and 16, were synthesized with a DNA synthesizer.

[0109] Using the chromosomal DNA as a template, PCR was performed with these primers and with TaKaRa LA-PCR™ Kit Ver.2 (produced by TAKARA), Expand™ High-Fidelity PCR System (produced by Boehringer Mannheim) or Taq DNA polymerase (produced by Boehringer) using a DNA Thermal Cycler (produced by Perkin-Elmer Japan).

[0110] PCR was performed for 30 cycles in which each cycle consists of reaction steps of 30 seconds at 94°C, 30 seconds at 55°C and 2 minutes at 72°C for DNA fragments of 2kb or less; and 20 seconds at 98°C, 3 minutes at 68°C

for DNA fragments of more than 2kb, and then reaction was carried out for 7min at 72°C.

[0111] Among DNA fragments amplified by PCR, the DNA fragment (containing *biol* gene) amplified by a combination of primers of SEQ ID NOS:3 and 4 was digested with restriction enzymes EcoRI and Sall. DNA fragment (containing *cypA* gene) amplified by a combination of primers of SEQ ID NOS:5 and 6 was digested with XbaI and SmaI. DNA fragment (containing *cypX* gene) amplified by a combination of primers of SEQ ID NOS:7 and 8 was digested with SmaI and Sall. DNA fragment (containing *pksS* gene) amplified by a combination of primers of SEQ ID NOS:9 and 10 was digested with EcoRI and Sall. DNA fragment (containing *yet0* gene) amplified by a combination of primers of SEQ ID NOS:11 and 12 was digested with XbaI and BglII. DNA fragment (containing *yjiB* gene) amplified by a combination of primers of SEQ ID NOS:13 and 14 was digested with XbaI and SmaI, and DNA fragment (containing *yrhJ* gene) amplified by a combination of primers of SEQ ID NOS:15 and 16 was digested with XbaI and SmaI, respectively.

[0112] After digestion, the DNA fragments treated with the restriction enzymes were subjected to agarose gel electrophoresis to obtain the DNA fragments treated with various restriction enzymes.

[0113] A vector plasmid pUC119 (produced by TAKARA) was digested with restriction enzymes Sall and EcoRI, then subjected to agarose gel electrophoresis to obtain a Sall-EcoRI treated pUC119 fragment. Similarly, a vector plasmid pUC119 was digested with restriction enzymes Sall and SmaI, then subjected to agarose gel electrophoresis to obtain a Sall-SmaI treated pUC119 fragment.

[0114] pSTV28 (produced by TAKARA) was digested with restriction enzymes XbaI and SmaI, then subjected to agarose gel electrophoresis to obtain a XbaI-SmaI treated pSTV28 fragment. Similarly, a vector plasmid pSTV28 was digested with restriction enzymes XbaI and BamHI, then subjected to agarose gel electrophoresis to obtain a XbaI-BamHI treated pSTV28 fragment.

[0115] The thus-obtained EcoRI-Sall treated DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:3 and 4) was mixed with the Sall-EcoRI treated pUC119 fragment. XbaI-SmaI treated DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:5 and 6) was mixed with the XbaI-SmaI treated pSTV28 fragment. SmaI-Sall treated DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:7 and 8) was mixed with the Sall-SmaI treated pUC119 fragment. EcoRI-Sall treated DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:9 and 10) was mixed with the Sall-EcoRI treated pUC119 fragment. XbaI-BglII treated DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:11 and 12) was mixed with the XbaI-BamHI treated pSTV28 fragment. XbaI-SmaI treated DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:13 and 14) was mixed with the XbaI-SmaI treated pSTV28 fragment. XbaI-SmaI treated DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:15 and 16) was mixed with XbaI-SmaI treated pSTV28 fragment, respectively. After ethanol precipitation, the obtained DNA precipitates were dissolved in 5μl of distilled water, and a ligation reaction was carried out to obtain each recombinant DNA.

[0116] Using the recombinant DNA, *E. coli* (purchased from TOYOB0) DH5α strain is transformed by a usual method, then the transformant was plated to a LB agar medium [containing Bacto Trypton (produced by Difco) 10g, Bacto yeast extract (produced by Difco) 5g, NaCl 5g in 1L; and adjusted to pH 7.4 with 1mol/l NaOH such that the agar is adjusted to 1.5%] containing 100μg/ml ampicillin in the case where the pUC119 is used as a vector plasmid; and to a LB agar medium containing 25μ g/ml chloramphenicol in the case where the pSTV28 is used as a vector plasmid, followed by culturing for 2 days at 25°C.

[0117] Several colonies of the grown ampicillin-resistant or chloramphenicol-resistant transformants were selected, inoculated in 10ml LB liquid medium [which contains Bacto Trypton (produced by Difco) 10g, Bacto yeast extract (produced by Difco) 10g and NaCl 5g in 1L; and is adjusted to pH 7.4 with 1mol/l NaOH], and then cultured while shaking for 2 days at 25°C.

[0118] The obtained culture was centrifuged to recover cells.

[0119] A plasmid was isolated from the cells in a usual manner.

[0120] The structure of the isolated plasmid was examined by cleaving it with various restriction enzymes and the nucleotide sequences were determined, thereby confirming that the desired DNA fragment was inserted in the plasmid. The plasmids obtained by linking the DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:3 and 4) treated with EcoRI-Sall to pUC119 fragment treated with Sall-EcoRI, was named pUbiol, the plasmids obtained by linking DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:5 and 6) treated with XbaI-SmaI to pSTV28 fragment treated with XbaI-SmaI was named pScypA, the plasmids obtained by linking DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:7 and 8) treated with SmaI-Sall to pUC119 fragment treated with Sall-SmaI was named pUcypX, the plasmids obtained by linking DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:9 and 10) treated with EcoRI-Sall to pUC119 fragment treated with Sall-EcoRI was named pUpksS, the plasmids obtained by linking DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:11 and 12) treated with XbaI-BglII to pSTV28 fragment treated with XbaI-BamHI was named pSyet0, the plasmids obtained by linking DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:13 and 14) treated with XbaI-SmaI to pSTV28 fragment treated with XbaI-SmaI was named pSyjB, the plasmids obtained by linking DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:15 and 16) treated with

XbaI-SmaI to pSTV28 fragment treated with XbaI-SmaI was named pSyrhJ, respectively.

[0121] *E.coli* DH5 α containing the thus-obtained plasmid, *E.coli* DH5 α containing pUC119 or pSTV28, and *E.coli* DH5 α containing no plasmid were inoculated respectively in 3ml of LB liquid medium (to which a drug which corresponds to a drug-resistant gene in a vector plasmid was added) and cultured while shaking for 12 hours at 28°C. The culture solution (0.5ml) was inoculated to a LB liquid medium (to which a drug which corresponds to a drug-resistant gene was added) containing 1% glucose and 1% CaCO_3 , and was cultured while shaking for 12 hours at 28°C. The culture solution (1ml) was poured into an assist tube (produced by ASSIST), then glucose and the previously obtained compound (VII-a) (wherein R¹ is a Na) were added to a final concentration of 1% and 100mg/l, respectively, followed by shaking for 24 hours at 28°C. Upon completion of the reaction, cells were removed by centrifugation, then the obtained supernatant was thoroughly shaken with addition of the same amount of ethyl acetate. The upper ethyl acetate layer was separated from the solution by centrifugation, then the ethyl acetate layer was evaporated to dryness by a centrifugal evaporator. The dried matter was dissolved in one-fifths volume of methanol relative to that of the first culture supernatant, and subjected to a HPLC analysis [column; Inertsil ODS-2 (5 μm , 4x250mm, manufactured by GL science), column temperature: 60°C, mobile phase: acetonitrile: water: phosphoric acid=55:45:0.05, flow rate: 0.9ml/min, detection wavelength: 237nm] to detect and quantify the compound (VIII-a) (wherein R¹ is Na). The results are shown in Table 1.

Table 1

Plasmid	Compound (VIII-a) (mg/l)
None	0
pUC119	0
pSTV28	0
pUbiol	0
pScypA	0
pUcypX	0
pUpksS	0
pYet0	0
pSyjIB	0.6
pSyrhJ	0

Example 2: Expression of yjiB gene in *Bacillus subtilis* as a host cell and confirmation of activity of the protein encoded by said gene

[0122] Sense and antisense primers having a combination of nucleotide sequences shown by SEQ ID NOS:17 and 18, SEQ ID NOS:19 and 20, SEQ ID NOS:21 and 22, SEQ ID NOS:23 and 24, SEQ ID NOS:25 and 26, SEQ ID NOS:27 and 28, and SEQ ID NOS:29 and 30, were synthesized with a DNA synthesizer.

[0123] Using the chromosomal DNA of *Bacillus subtilis* obtained in Example 1 as a template, PCR was performed with these primers and with TaKaRa LA-PCR™ Kit Ver.2 (produced by TAKARA), Expand™ High-FidelityPCR System (produced by Boehringer Mannheim) or Taq DNA polymerase (produced by Boellinnger) using a DNA Thermal Cycler (produced by Perkin-Elmer Japan).

[0124] PCR was performed for 30 cycles under the conditions where one cycle consists of the reaction steps of 30 seconds at 94°C, 30 seconds at 55°C and 2 minutes at 72°C for the DNA fragments of 2kb or less, and 20 seconds at 98°C and 3 minutes at 68°C for the DNA fragments of more than 2kb, and then a reaction was carried out for 7 minutes at 72°C.

[0125] Among DNA fragments amplified by PCR, the DNA fragment (containing biol gene) amplified by a combination of primers of SEQ ID NOS:17 and 18 was digested with restriction enzymes SpeI and BamHI, DNA fragment (containing cypA gene) amplified by a combination of primers of SEQ ID NOS:19 and 20 was digested with SpeI and BamHI, DNA fragment (containing cypX gene) amplified by a combination of primers of SEQ ID NOS:21 and 22 was digested with SpeI and NruI, DNA fragment (containing pksS gene) amplified by a combination of primers of SEQ ID NOS:23 and 24 was digested with SpeI and BamHI, DNA fragment (containing yet0 gene) amplified by a combination of primers of SEQ ID NOS:25 and 26 was digested with SpeI and BamHI, DNA fragment (containing yjiB gene) amplified by a combination of primers of SEQ ID NOS:27 and 28 was digested with SpeI and BamHI, DNA fragment (containing yrhJ gene) amplified by a combination of primers of SEQ ID NOS:29 and 30 was digested with SpeI and BamHI, respectively.

[0126] After digestion, the DNA fragments treated with the restriction enzymes were subjected to agarose gel electrophoresis to obtain the DNA fragments treated with each restriction enzyme.

[0127] A vector plasmid pWH1520 (produced by MoBiTec) was digested with restriction enzymes SpeI and BamHI.

then subjected to agarose gel electrophoresis to obtain a SpeI-BamHI treated pWH1520 fragment. Similarly, a vector plasmid pWH1520 was digested with restriction enzymes SpeI and NruI, then subjected to agarose gel electrophoresis to obtain a SpeI-NruI pWH1520 fragment.

[0128] The thus-obtained SpeI-BamHI treated DNA fragments (amplified by PCR with a combination of primers of SEQ ID NOS:17 and 18, SEQ ID NOS:19 and 20, SEQ ID NOS:23 and 24, SEQ ID NOS:25 and 26, SEQ ID NOS:27 and 28, and SEQ ID NOS:29 and 30) were mixed with the SpeI-BamHI treated pWF1520 fragment; SpeI-NruI treated DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:21 and 22) was mixed with SpeI-NruI pWF1520 fragment, respectively. After ethanol precipitation, the obtained DNA precipitates were dissolved in 5µl of distilled water, and a ligation reaction was carried out to obtain each recombinant DNA.

[0129] Using the recombinant DNA, *E.coli* (purchased from TOYOB0) DH5 α strain was transformed by a usual method, then plated to a LB agar medium containing 10µ g/ml of tetracycline, and cultured for 2 days at 25°C. Cells were recovered from the obtained culture by centrifugation.

[0130] A plasmid was isolated from the cells in a usual manner.

[0131] The structure of the isolated plasmid was examined by cleaving it with various restriction enzymes and the nucleotide sequences thereof were determined, thereby confirming that the desired DNA fragment was inserted in the plasmid. The plasmid obtained by linking the DNA fragment amplified by PCR with a combination of primers of SEQ ID NOS:17 and 18 to pWH1520 was named as pWbiol; the plasmid obtained by linking the DNA fragment amplified by PCR with a combination of primers of SEQ ID NOS:19 and 20 to pWH1520 was named as pWcypA; the plasmid obtained by linking the DNA fragment amplified by PCR with a combination of primers of SEQ ID NOS:21 and 22 to pWH1520 was named as pWcypX; the plasmid obtained by linking the DNA fragment amplified by PCR with a combination of primers of SEQ ID NOS:23 and 24 to pWH1520 was named as pWpkS; the plasmid obtained by linking the DNA fragment amplified by PCR with a combination of primers of SEQ ID NOS:25 and 26 to pWH1520 was named as pWyetO; the plasmid obtained by linking the DNA fragment amplified by PCR with a combination of primers of SEQ ID NOS:27 and 28 to pWH1520 was named as pWyiB; the plasmid obtained by linking the DNA fragment amplified by PCR with a combination of primers of SEQ ID NOS:29 and 30 to pWH1520 was named as pWyrhJ, respectively.

[0132] The thus-obtained plasmids and the vector plasmid pWH1520 were introduced in a *Bacillus subtilis* ATCC33712 strain according to the method by S.chang and S.N. cohen [S. chang and S.N. cohen: Mol. Gen. Genet., 168, 111 (1979).]

[0133] That is, ATCC33712 strain was inoculated in a thick tube containing 5ml of Pen medium (where 1.75 g of Difco Antibiotic medium No. 3 was dissolved in 100ml of water and sterilized in an autoclave), then cultured with shaking at 37°C overnight. Total cells cultured overnight in 300ml Erlenmeyer flask-containing 100ml of Pen medium were then inoculated and cultured with shaking for 3 hours at 37°C to be grown until reaching a metaphase of exponential growth. The culture was centrifuged for 10 minutes at 5000rpm in germ-free conditions to precipitate the cells. After removing the supernatant, the cells were suspended in 4.5ml of SMMP [mixture comprising equal amount of 2 x SMMP (where sucrose 34.2g, maleic acid 0.464g, magnesium chloride-6H₂O 0.813g were dissolved in water, which was adjusted to pH6.5 with sodium hydroxide, then the final volume of 100ml was sterilized in an autoclave) and 4 x Pen medium (where 7g of Difco Antibiotic medium No. 3 was dissolved in 100ml of water and sterilized in an autoclave)], followed by addition of 0.5ml of lysozyme solution [where 10mg of lysozyme (produced by EIKAGAKU corp.) was dissolved in 0.5ml of SMMP, and sterilized with a millipore filter having a pore size of 0.45µm], and the mixture was slowly shaken for 2 hours at 37°C. After microscopically confirming that not less than 90% cells became protoplast, the protoplasts were centrifuged for 20 minutes at 3000rpm to be precipitated. The supernatant was removed, and the obtained protoplasts were resuspended in 5 ml of SMMP. The protoplasts were collected by centrifugation for 20 minutes at 3000 rpm, and suspended in 2ml of SMMP to prepare a protoplast suspension of a recipient strain for transformation.

[0134] Approximately 1µg of plasmid DNA was dissolved in SMMP, and thoroughly mixed with 0.5ml of protoplast suspension. Immediately after mixing, 1.5ml of 40% polyethylene glycol solution [where 40g of polyethylene glycol 6000 (Nacalai tesque) was dissolved in 2 x SMMP, and water was added thereto to become the volume of 100ml, followed by sterilization in an autoclave] was added thereto and thoroughly mixed. After standing at room temperature for 2 minutes, 5ml of SMMP was added and mixed, and the mixture was centrifuged for 20 minutes at 3000rpm. After removing the supernatant, the precipitated protoplasts were suspended in 1ml of SMMP, then slowly shaken for 3 hours at 30°C. After dilution with SMMP as appropriate, the protoplasts were applied to a DM3 medium [in which 45ml of 80g/L bactoagar (produced by Difco), 50ml of 50g/L casamino acid, 250ml of 338g/L sodium succinate-6H₂O (pH7.3), 50ml phosphate buffer (35g/L potassium hydrogen phosphate, 15g/L potassium dihydrogen phosphate), 25ml of 100g/L yeast extract, 10ml of 203g/L magnesium chloride-6H₂O, 25ml of 100g/L glucose were respectively sterilized in an autoclave and mixed, then 3.5ml of 20mg/ml bovine serum albumin sterilized with millipore filter having a pore size of 0.45µm was added thereto] wherein the medium containing drugs (in case of tetracycline, it was added to the final volume of 10µg/ml). The protoplasts were cultured for 1 to 2 days at 37°C to obtain the transfected strain.

[0135] Thus, *B. subtilis* ATCC33712 strains having each of the above plasmids were obtained.

[0136] The obtained transformants and ATCC33712 strain having no plasmid were inoculated respectively in 3ml

LB liquid media (wherein 10mg/l tetracycline was added to a plasmid-containing strain), and cultured with shaking for 24 hours at 30°C. 0.25ml of this culture solution was inoculated in a test tube containing a 5ml of TB medium [Bacto Trypton (produced by Difco) 1.4%, Bacto yeast extract (produced by Difco) 2.4%, KH₂PO₄ 0.231%, and K₂HPO₄ 1.251%, adjusted to pH7.4 with 1mol/l sodium hydroxide], and cultured with shaking for 3 hours at 30°C. After 3 hours, 5 1ml of the culture was transferred to an assist tube No. 60.540S (produced by ASSIST) and 40μl of 50% sterilized xylose solution was added thereto, followed by culturing with shaking for 3 hours. Then, the compound (VII-a) (wherein R is Na) obtained in Example 1 was added to each test tube to the final concentration of 0.2mg/ml, and the mixture was cultured with shaking for 16 hours at 30°C.

10 [0137] Upon completion of reaction, the reaction solution was adjusted to pH 3.5 with acetic acid. 1ml of ethyl acetate was added to 0.5ml of this reaction solution, and the mixture was shaken for 1 hour. After shaking, the reaction solution was centrifuged for 5 minutes at 3000rpm to be separated into 2 layers, and the upper ethyl acetate layer was recovered, the solvent was removed by a centrifugal evaporator, and the residue was dissolved in 0.5ml of methanol.

15 [0138] Using an aliquot of this methanol solution, HPLC analysis was performed as in Example 1 to detect and quantify compound (VIII-a) (wherein R¹ is Na). The results are shown in Table 2.

Table 2

Plasmid	Compound (VIII-a) (mg/l)
None	0.5
pWH1520	0.5
pWbiol	0.5
pWcypA	0.5
pWcypX	0.5
pWpksS	0.5
pWyetO	0.5
pWyjiB	24.6
pWyrhJ	0.5

20 [0139] As seen from the results of Example 1 and 2, it is obvious that activity of producing compound (VIII-a) or compound (VIII-b) from compound (VII-a) or compound (VII-b) is encoded by yjiB gene.

25 [0140] The DNA fragment amplified by PCR with a combination of primers of SEQ ID NO:27 and 28 above, contained the nucleotide sequence shown by SEQ ID NO:2; and said nucleotide sequence contained a nucleotide sequence encoding a protein having the amino acid sequence shown by SEQ ID NO:1.

30 [0141] pWyjiB prepared in Example 2 was introduced into *Bacillus megaterium* (produced by MoBiTec) and *Bacillus* sp. FERM BP-6030 in the same manner as is described for transformation of *Bacillus subtilis* in Example 2.

35 [0142] The obtained transformant and a host cell having no plasmid were cultured and reaction was carried out in the same manner as in Example 2, and the amount of produced compound (VIII-a) was measured. The results are shown in Table 3.

Table 3

Host	Plasmid	Compound (VIII-a) (mg/l)
<i>B. megaterium</i>	none	2.0
(as above)	pWyjiB	27.2
FERM BP-6030	none	4.5
(as above)	pWyjiB	30.3

45 Example 4: Preparation of the plasmid for expressing the protein which produces compound (VIII-a) in coryne-form bacteria

50 [0143] To allow efficient expression of yjiB gene obtained in Example 1 in coryne-form bacteria, DNAs having nucleotide sequences shown by SEQ ID NOS:31, 32, 33, 34, 35, 36, 37, 38 and 39 were synthesized with a DNA synthesizer.

55 [0144] The plasmid pR1109 DNA in which the DNA fragment comprising a promoter sequence p54-6 (GenBank AJ1325B2) for expression in coryne-form bacteria and having the nucleotide sequence shown by SEQ ID NO:40 was

inserted into a Sse83871-BamHI site of a plasmid vector pCS299P (Japanese Patent Application No. 11-110437), was prepared in a usual manner from E.coli NM522 strain transformed with this plasmid.

[0145] Using pWYjiB DNA obtained in Example 2 as a template, PCR was performed with DNA primers having nucleotide sequences shown by SEQ ID NOS:31 and 32 and with Taq DNA polymerase (produced by TAKARA) using a DNA Thermal Cycler 480 (produced by Perkin-Elmer Japan).

[0146] PCR was performed for 25 cycles in which each cycle consists of reaction steps of 30 seconds at 96°C, 45 seconds at 50°C and 3 minutes at 72°C.

[0147] DNA fragment amplified by PCR was digested with Sall and BamHI and subjected to agarose gel electrophoresis, and an approximately 1.2kb DNA fragment was purified in a usual manner to obtain a Sall-BamHI treated DNA fragment.

[0148] The above-obtained plasmid pRI109 DNA was digested with restriction enzymes Sall and BamHI and subjected to agarose gel electrophoresis, and an approximately 6 kb DNA fragment was purified in a usual manner to obtain a Sall-BamHI treated pRI109 fragment.

[0149] The above-obtained Sall-BamHI treated DNA fragment and Sall-BamHI treated pRI109 fragment were mixed, and ligation reaction was carried out to obtain the recombinant DNA.

[0150] Using the recombinant DNA, E.coli DH5 α (purchased from TOYOB) was transformed by a usual method, then plated to a LB agar medium containing 20 μ g/ml kanamycin and cultured for 1 day at 30°C to obtain the transformant.

[0151] A plasmid was isolated from the transformant in a usual manner. Using the isolated plasmid DNA as a template, and using DNAs having nucleotide sequences shown by SEQ ID NOS:33, 34, 35, 36 and 37 as primers respectively, the nucleotide sequences of the inserted DNA fragments were determined with a DyeTerminator Cycle Sequencing Kit (produced by Applied Biosystem) and 373A sequencer (produced by Applied Biosystem), then the plasmid in which the nucleotide sequence shown by SEQ ID NO:41 was inserted between Sall and BamHI sites of pRI109 was named pRlyjiB.

[0152] The nucleotide sequence shown by SEQ ID NO:41 contained the nucleotide sequence which encoded the protein having the amino acid sequence shown by SEQ ID NO:42.

[0153] Using the chromosomal DNA of *Bacillus subtilis* Marburg168 strain (ATCC15563) obtained in Example 1 as a template, PCR was performed with DNA primers having nucleotide sequences shown by SEQ ID NOS:38 and 39, and with LA-Taq DNA polymerase (produced by TAKARA) using a DNA Thermal Cycler 480 (produced by Perkin-Elmer Japan).

[0154] PCR was performed for 30 cycles in which each cycle consists of reaction steps of 30 seconds at 96°C, 30 seconds at 55°C and 2 minutes at 72°C, and then a reaction was carried out for 7 minutes at 72°C.

[0155] The DNA fragment amplified by PCR was mixed with pT7Blue (produced by TAKARA), and ligation reaction was carried out to obtain the recombinant DNA.

[0156] Using the recombinant DNA, E.coli DH5 α (purchased from TOYOB) was transformed by a usual method, then plated to a LB agar medium containing 100 μ g/ml ampicillin and cultured for 1 day at 30°C to obtain the transformant.

[0157] A plasmid was isolated from the transformant by a usual method. The structure of the isolated plasmid was examined by cleaving it with various restriction enzymes, thereby confirming that the desired DNA fragment was inserted in the plasmid, and the plasmid was named as pTSYN2-72.

[0158] The pTSYN2-72 DNA was digested with Xhol and BamHI and subjected to agarose gel electrophoresis, and then an approximately 1.2kb DNA fragment was purified by a usual method to obtain a Xhol-BamHI treated DNA fragment.

[0159] The plasmid pR1109 DNA was digested with restriction enzymes Sall and BamHI and subjected to agarose gel electrophoresis, and then an approximately 6kb DNA fragment was purified by a usual method to obtain a Sall-BamHI treated pRI109 fragment.

[0160] The above-obtained Xhol-BamHI treated DNA fragment and Sall-BamHI treated pRI109 fragment were mixed, and the ligation reaction was carried out to obtain the recombinant DNA.

[0161] Using the recombinant DNA, E.coli DH5 α (purchased from TOYOB) was transformed by a usual method, then plated to a LB agar medium containing 20 μ g/ml kanamycin and cultured for 1 day at 30°C to obtain a transformant.

[0162] A plasmid was isolated from the transformant by a usual method. Using the isolated plasmid DNA as a template, and using DNAs having nucleotide sequences shown by SEQ ID NOS:33, 34, 35, 36 and 37, the nucleotide sequences of the inserted DNA fragment were determined with a DyeTerminator Cycle Sequencing Kit (produced by Applied Biosystem) and 373A sequencer (produced by Applied Biosystem), and the plasmid in which the nucleotide sequence shown by SEQ ID NO:43 was inserted between Sall-BamHI site of pRI109, was named pSYN2-72.

[0163] The nucleotide sequence shown by SEQ ID NO:43 contained the nucleotide sequence which encodes the protein having the amino acid sequence shown by SEQ ID NO:1.

[0164] Using pWYjiB DNA obtained in Example 2 as a template, PCR was performed with DNA primers having nu-

cleotide sequences shown by SEQ ID NO:38 and 39. and with Z-Taq DNA polymerase (produced by TAKARA) using a DNA Thermal Cycler 480 (produced by Perkin-Elmer Japan).

[0165] PCR was performed for 25 cycles in which each cycle consists of reaction steps of 20 seconds at 98°C. 20 seconds at 55°C and 30 minutes at 72°C.

5 [0166] The DNA fragment amplified by PCR was digested with XbaI and BamHI and subjected to agarose gel electrophoresis. and then an approximately 1.2kb DNA fragment was purified by a usual method to obtain a XbaI-BamHI treated DNA fragment.

10 [0167] The plasmid pRI109 DNA was digested with restriction enzymes SalI and BamHI and subjected to agarose gel electrophoresis. then an approximately 6kb DNA fragment was purified by a usual method to obtain a SalI-BamHI treated pRI109 fragment.

[0168] The above-obtained XbaI-BamHI treated DNA fragment and SalI-BamHI treated pRI109 fragment were mixed. and ligation reaction was carried out to obtain the recombinant DNA.

15 [0169] Using the recombinant DNA, *E.coli* DH5 α (purchased from TOYOB) was transformed by a usual method. then plated to a LB agar medium containing 20 μ g/ml kanamycin and cultured for 1 day at 30°C to obtain the transformant.

20 [0170] A plasmid was isolated from the transformant by a usual method. Using the isolated plasmid DNA as a template, and using DNAs having nucleotide-sequences shown by SEQ ID NOS:33, 34, 35, 36 and 37 respectively as primers, the nucleotide sequences of the inserted DNA fragments were determined with a DyeTerminator Cycle Sequencing Kit (produced by Applied Biosystem) and 373A sequencer (produced by Applied Biosystem). and the plasmid in which the nucleotide sequence shown by SEQ ID NO:44 was inserted between SalI-BamHI site of pRI109. was named pSYN2-39.

25 [0171] The nucleotide sequence shown by SEQ ID NO:44 contained the nucleotide sequence which encodes the protein having the amino acid sequence shown by SEQ ID NO:45.

25 Example 5: Introduction of the plasmid into the *C. glutamicum* ATCC13032 strain and evaluation of activity

30 [0172] ATCC13032 strain was inoculated in a test tube containing 8ml of broth medium [20g/l normal broth medium (produced by Kyokuto Pharmaceutical Industry, Co. Ltd). 5g/l Bacto Yeast Extract (produced by Difco)] and cultured with shaking 30°C overnight. Subsequently, 5ml of cells cultured overnight were inoculated in a 2L Erlenmeyer flask (bearing a bubble(s)) containing 250ml of broth medium and cultured with shaking for 4 hours at 30°C. The obtained culture solution was centrifuged to precipitate the cells. After removing the supernatant. the cells were suspended in 30ml of ice-cold EPB [250mmol/l Sucrose, 15%(v/v) glycerol]. and centrifuged to be precipitated. Similarly. the cells were resuspended in EPB and centrifuged to be separated, and then the cells were suspended in 2ml of EPB. The obtained cell suspension was poured into 0.5ml tubes by 0.1ml each, and was quickly frozen with dry ice to obtain the cell suspension for transformation. The obtained cells were stored at a temperature below -80°C.

35 [0173] 0.1ml of the frozen cell suspension for transformation was dissolved on ice. retained for 10 minutes at 43.5°C. and transferred onto ice. After 2 μ l of aqueous solution containing approximately 2 μ g pRI109 DNA was added. the cell suspension was transferred to the previously iced *E.coli* GenePulser cuvet (produced by BioRad). and then the DNA was introduced into cells under conditions of 25 μ F, 200 Ω and 1.5kV by electroporation using GenePulser (produced by BioRad). Immediately after electroporation, total amount of the cell suspension was moved to a 15ml-test tube containing 1ml of broth medium. and cultured with shaking for 1 hour at 30°C.

40 [0174] The obtained culture solution was centrifuged for 10 minutes at 3,500rpm to precipitate the cells. After removing the supernatant. the cells were suspended with addition of 0.1ml broth medium. then the suspension was applied to a broth agar medium [which was solidified with 2% Difco Agar] containing 20 μ g/ml kanamycin and cultured for 2 days at 30°C to obtain the transformant.

45 [0175] Thus, *C.g*lutamicum ATCC13032 strain having pRI109 was obtained.

[0176] As in the above, *C.g*lutamicum ATCC13032 strains having each plasmid, pRlyjB, pSYN2-72, pSYN2-39 were obtained.

50 [0177] The obtained transformants were inoculated in test tubes which contain 3ml of broth media containing 100 μ g/ml kanamycin, and cultured with shaking for 24 hours at 30°C. The culture (0.2 ml) was inoculated in a test tube containing 2ml of LMC medium [in which separately sterilized Glucose, MgSO₄, FeSO₄, MnSO₄ were added to a pre-LMC medium sterilized in a autoclave (NH₄Cl 1g/l, KH₂PO₄ 1g/l, K₂HPO₄ 3g/l, Difco Yeast Extract 0.2g/l, Urea 1g/l, Biotin 0.05mg/l, Thiamin 0.5mg/l, Corn Steep Liquor 10g/l; pH7.2) to the final concentration of 30g/l, 0.1g/l, 2mg/l and 2mg/l, respectively] wherein the medium contains 100 μ g/ml kanamycin, and cultured with shaking for 5 hours at 30°C. The compound (VII-a) (wherein R is Na) was added thereto to the final concentration of 300mg/l. and the mixture was reacted with shaking for 16 hours at 30°C.

55 [0178] 0.5ml of the reaction solution was moved to a 1.5ml tube. and centrifuged for 2 minutes at 15,000rpm to separate the cells. The obtained supernatant was diluted 5 to 20 times with methanol and centrifuged for 2 minutes at

15,000rpm, and then an aliquot thereof was used for HPLC analysis as in Example 1 to detect and quantify the compound (VIII-a) (wherein R¹ is Na). The concentration of the compound (VIII-a) in the reaction solution calculated based on the quantification result, is shown in Table 4.

5

Table 4

10

Plasmid	Compound (VIII-a)(mg/l)
pRI109	0.3
pSYN2-72	30
pRlyjiB	61
pSYN2-39	104

Example 6: Introduction of the plasmid into coryne-form bacteria and evaluation of activity

15 [0179] pRlyjiB DNA obtained in Example 4 was introduced into *C.callunae* ATCC15991, *C.ammoniagenes* ATCC6872 and *B.flavum* ATCC14067 in the same manner as in the transformation of ATCC13032 strain described in Example 5, and transformants were obtained from each strain.

20 [0180] The obtained transformants were respectively inoculated on 3ml of broth media in test tubes containing 100µg/ml kanamycin, and cultured with shaking for 24 hours at 30°C. The culture (0.5ml) was transferred to a test tube containing 5ml TB medium [in which 14g of Bacto Trypton (produced by Difco) and 24g of Bacto Yeast Extract (produced by Difco) were dissolved in 900ml of water and sterilized in an autoclave, to which 100ml PB [KH₂PO₄ 23.1g/l, K₂HPO₄ 125.1g/l] separately sterilized in an autoclave was added] wherein the medium contains 100µg/ml kanamycin and 10g/l Glucose, and cultured with shaking for 5 hours at 30°C. The culture (1ml) was transferred to an assist tube (produced by ASSIST), and compound (VII-a) (wherein R is Na) was added thereto to the final concentration of 300mg/l, and the mixture was reacted with shaking for 16 hours at 30°C.

25 [0181] Upon completion of reaction, compound (VIII-a) (wherein R¹ is Na) in the culture was detected and quantified in the method as in Example 2. The concentration of compound (VIII-a) in the culture calculated based on the quantification results, is shown in Table 5.

30

Table 5

35

Host Cell	Plasmid	Compound (VIII-a) (mg/l)
<i>C.callunae</i> ATCC15991 (KY3510)	pRlyjiB	22
<i>C.ammoniagenes</i> ATCC6872 (KY3454)	pRlyjiB	12
<i>B.flavum</i> ATCC14067 (KY10122)	pRlyjiB	23

Industrial Applicability

40 [0182] The present invention enables efficient production of a DNA encoding a novel hydroxylase and a compound inhibiting hydroxymethylglutaryl CoA (HMG-CoA) reductase and has an action of reducing serum cholesterol.

Free Text of Sequence Listing

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EP 1 148 122 A1

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 10 aaa gca tgg tct gat ctt ctg gtc agt aca ccg aag gat aaa agt gaa 529
 Lys Ala Trp Ser Asp Leu Leu Val Ser Thr Pro Lys Asp Lys Ser Glu
 160 165 170

 15 gaa gct gaa aaa gcc ttt ttg gaa gaa cga gat aag tgt gag gaa gaa 577
 Glu Ala Glu Lys Ala Phe Leu Glu Glu Arg Asp Lys Cys Glu Glu Glu
 175 180 185 190

 20 ctg gcc gcg ttt ttt gcc ggc atc ata gaa gaa aag cga aac aaa ccg 625
 Leu Ala Ala Phe Phe Ala Gly Ile Ile Glu Glu Lys Arg Asn Lys Pro
 195 200 205

 25 gaa cag gat att att tct att tta gtg gaa gcg gaa gaa aca ggc gag 673
 Glu Glu Asp Ile Ile Ser Ile Leu Val Glu Ala Glu Glu Thr Gly Glu
 210 215 220

 30 aag ctg tcc ggt gaa gag ctg att ccg ttg tgc acg ctg ctg ctg gtg 721
 Lys Leu Ser Gly Glu Glu Leu Ile Pro Leu Cys Thr Leu Leu Leu Val
 225 230 235

 35 gcc gga aat gaa acc act aca aac ctg att tca aat gcg atg tac agc 769
 Ala Gly Asn Glu Thr Thr Asp Leu Ile Ser Asn Ala Met Tyr Ser
 240 245 250

 40 ata tta gaa acg cca ggc gtt tac gaa gaa ctg cgc agc cat cct gaa 817

	Ile Leu Glu Thr Pro Gly Val Tyr Glu Glu Leu Arg Ser His Pro Glu			
5	255	260	265	270
	ctg atg cct cag gca gtg gag gaa gcc ttg cgt ttc aga gcg ccg gcc	865		
10	Leu Met Pro Gln Ala Val Glu Glu Ala Leu Arg Phe Arg Ala Pro Ala			
	275	280	285	
15	ccg gtt ttg agg cgc att gcc aag cgg gat acg gag atc ggg ggg cac	913		
	Pro Val Leu Arg Arg Ile Ala Lys Arg Asp Thr Glu Ile Gly Gly His			
20	290	295	300	
	ctg att aaa gaa ggt gat atg gtt ttg gcg ttt gtg gca tcg gca aat	961		
25	Leu Ile Lys Glu Gly Asp Met Val Leu Ala Phe Val Ala Ser Ala Asn			
	305	310	315	
30	cgt gat gaa gca aag ttt gac aga ccg cac atg ttt gat atc cgc cgc	1009		
	Arg Asp Glu Ala Lys Phe Asp Arg Pro His Met Phe Asp Ile Arg Arg			
35	320	325	330	
	cat ccc aat ccg cat att gcg ttt ggc cac ggc atc cat ttt tgc ctt	1057		
40	His Pro Asn Pro His Ile Ala Phe Gly His Gly Ile His Phe Cys Leu			
	335	340	345	350
45	ggg gcc ccg ctt gcc cgt ctt gaa gca aat atc gcg tta acg tct ttg	1105		
	Gly Ala Pro Leu Ala Arg Leu Glu Ala Asn Ile Ala Leu Thr Ser Leu			
50	355	360	365	
	att tct gct ttt cct cat atg gag tgc gtc agt atc act ccg att gaa	1153		
55	Ile Ser Ala Phe Pro His Met Glu Cys Val Ser Ile Thr Pro Ile Glu			

370

375

380

5

aac agt gtg ata tac gga tta aag agc ttc cgt gtg aaa atg taaggatcc 1204
 Asn Ser Val Ile Tyr Gly Leu Lys Ser Phe Arg Val Lys Met

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<210> 42

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<212> PRT

<213> Bacillus subtilis

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<400> 42

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Gly Lys Asn Lys Gln Asp Ala Tyr His Pro Phe Pro Trp Tyr Glu Ser

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Met Arg Lys Asp Ala Pro Val Ser Phe Asp Glu Glu Asn Gln Val Trp

35

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Ser Val Phe Leu Tyr Asp Asp Val Lys Lys Val Val Gly Asp Lys Glu

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Leu Phe Ser Ser Cys Met Pro Gln Gln Thr Ser Ser Ile Gly Asn Ser

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Ile Ile Asn Met Asp Pro Pro Lys His Thr Lys Ile Arg Ser Val Val
 5 85 90 95

Asn Lys Ala Phe Thr Pro Arg Ala Met Lys Gln Trp Glu Pro Arg Ile
 10 100 105 110

Gln Glu Ile Thr Asp Glu Leu Ile Gln Lys Phe Gln Gly Arg Ser Glu
 15 115 120 125

Phe Asp Leu Val His Asp Phe Ser Tyr Pro Leu Pro Val Ile Val Ile
 20 130 135 140

Ser Glu Leu Leu Gly Val Pro Ser Ala His Met Glu Gln Phe Lys Ala
 25 145 150 155 160

Trp Ser Asp Leu Leu Val Ser Thr Pro Lys Asp Lys Ser Glu Glu Ala
 30 165 170 175

Glu Lys Ala Phe Leu Glu Glu Arg Asp Lys Cys Glu Glu Glu Leu Ala
 35 180 185 190

Ala Phe Phe Ala Gly Ile Ile Glu Glu Lys Arg Asn Lys Pro Glu Gln
 40 195 200 205

Asp Ile Ile Ser Ile Leu Val Glu Ala Glu Glu Thr Gly Glu Lys Leu
 45 210 215 220

Ser Gly Glu Glu Leu Ile Pro Leu Cys Thr Leu Leu Leu Val Ala Gly
 50 225 230 235 240

5 Asn Glu Thr Thr Thr Asn Leu Ile Ser Asp Ala Met Tyr Ser Ile Leu
245 250 255

10 Glu Thr Pro Gly Val Tyr Glu Glu Leu Arg Ser His Pro Glu Leu Met
260 265 270

15 Pro Gln Ala Val Glu Glu Ala Leu Arg Phe Arg Ala Pro Ala Pro Val
275 280 285

20 Leu Arg Arg Ile Ala Lys Arg Asp Thr Glu Ile Gly Gly His Leu Ile
290 295 300

25 Lys Glu Gly Asp Met Val Leu Ala Phe Val Ala Ser Ala Asp Arg Asp
305 310 315 320

30 35 Glu Ala Lys Phe Asp Arg Pro His Met Phe Asp Ile Arg Arg His Pro
325 330 335

40 Asn Pro His Ile Ala Phe Gly His Gly Ile His Phe Cys Leu Gly Ala
340 345 350

45 Pro Leu Ala Arg Leu Glu Ala Asn Ile Ala Leu Thr Ser Leu Ile Ser
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50 Ala Phe Pro His Met Glu Cys Val Ser Ile Thr Pro Ile Glu Asn Ser
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55 Val Ile Tyr Gly Leu Lys Ser Phe Arg Val Lys Met

385

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<210> 43

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<212> DNA

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<213> *Bacillus subtilis*

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35

tig cag cga gcg ctg ctc aat ggg aaa aac aaa cag gat gcg tat cat 99
 Leu Gln Arg Ala Leu Leu Asn Gly Lys Asn Lys Gln Asp Ala Tyr His

40

10 15 20 25

45

ccg ttt cca tgg tat gaa tcg atg aga aag gat gcg cct gtt tcc ttt 147
 Pro Phe Pro Trp Tyr Glu Ser Met Arg Lys Asp Ala Pro Val Ser Phe

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gat gaa gaa aac caa gtg tgg agc gti ttt ctt tat gat gat gtc zaa 195
 Asp Glu Glu Asn Gln Val Trp Ser Val Phe Leu Tyr Asp Asp Val Lys

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5 aaa gtt gtt ggg gat aaa gag ttg ttt tcc agt tgc atg ccc cag cag 243
 Lys Val Val Gly Asp Lys Glu Leu Phe Ser Ser Cys Met Pro Gln Gln
 60 65 70

 10 aca agc tct att gga aat tcc atc att aac atg gac ccc ccc aag cat 291
 Thr Ser Ser Ile Gly Asn Ser Ile Ile Asn Met Asp Pro Pro Lys His
 15 75 80 85

 20 aca aaa atc cgt tca gtc gtg aac aaa gcc ttt act ccc ccc gtg atg 339
 Thr Lys Ile Arg Ser Val Val Asn Lys Ala Phe Thr Pro Arg Val Met
 25 90 95 100 105

 30 aag caa tgg gaa ccc aga att caa gaa atc aca gat gaa ctg att caa 387
 Lys Gln Trp Glu Pro Arg Ile Gln Glu Ile Thr Asp Glu Leu Ile Gln
 35 110 115 120

 40 aaa ttt cag ggg ccc agt gag ttt gac ctt gtt cac gat ttt tca tac 435
 Lys Phe Gln Gly Arg Ser Glu Phe Asp Leu Val His Asp Phe Ser Tyr
 45 125 130 135

 50 ccg ctt ccg gtt att gtg ata tct gag ctg ctg gga gtg cct tca ccc 483
 Pro Leu Pro Val Ile Val Ile Ser Glu Leu Leu Gly Val Pro Ser Ala
 55 140 145 150

 60 cat atg gaa cag ttt aac gca tgg tct gat ctt ctg gtc agt aca ccc 531
 His Met Glu Gln Phe Lys Ala Trp Ser Asp Leu Leu Val Ser Thr Pro
 65 155 160 165

aag gat aaa agt gaa gaa gct gaa aaa gcc ttt ttg gaa gaa cga gat 579
 Lys Asp Lys Ser Glu Glu Ala Glu Lys Ala Phe Leu Glu Glu Arg Asp
 5 170 175 180 185

10 aag tgt gag gaa gaa ctg gcc gcg ttt ttt gcc ggc atc ata gaa gaa 627
 Lys Cys Glu Glu Glu Leu Ala Ala Phe Phe Ala Gly Ile Ile Glu Glu
 190 195 200

15 aag cga aac aaa ccg gaa cag gat att att tct att tta gtg gaa gcg 675
 Lys Arg Asn Lys Pro Glu Gln Asp Ile Ile Ser Ile Leu Val Glu Ala
 20 205 210 215

25 gaa gaa aca ggc gag aag ctg tcc ggt gaa gag ctg att ccg ttt tgc 723
 Glu Glu Thr Gly Glu Lys Leu Ser Gly Glu Glu Leu Ile Pro Phe Cys
 220 225 230

30 acg ctg ctg ctg gtg gcc gga aat gaa acc act aca aac ctg att tca 771
 Thr Leu Leu Leu Val Ala Gly Asn Glu Thr Thr Asn Leu Ile Ser
 35 235 240 245

40 aat gcg atg tac agc ata tta gaa acg cca ggc gtt tac gag gaa ctg 819
 Asn Ala Met Tyr Ser Ile Leu Glu Thr Pro Gly Val Tyr Glu Glu Leu
 250 255 260 265

45 cgc agc cat cct gaa ctg atg cct cag gca gtg gag gaa gcc ttg cgt 867
 Arg Ser His Pro Glu Leu Met Pro Gln Ala Val Glu Glu Ala Leu Arg
 50 270 275 280

55 ttc aga gcg ccg gcc ccg gtt ttg agg cgc att gcc aag cgg gat acg 915

EP 1 148 122 A1

Phe Arg Ala Pro Ala Pro Val Leu Arg Arg Ile Ala Lys Arg Asp Thr
 5 285 290 295

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 10 Glu Ile Gly Gly His Leu Ile Lys Glu Gly Asp Met Val Leu Ala Phe
 300 305 310

15 gtg gca tcg gca aat cgt gat gaa gca aag ttt gac aga ccg cac atg 1011
 Val Ala Ser Ala Asn Arg Asp Glu Ala Lys Phe Asp Arg Pro His Met
 20 315 320 325

ttt gat atc cgc cgc cat ccc aat ccg cat att gcg ttt ggc cac ggc 1059
 25 Phe Asp Ile Arg Arg His Pro Asn Pro His Ile Ala Phe Gly His Gly
 330 335 340 345

30 atc cat ttt tgc ctt ggg gcc ccg ctt gcc cgt ctt gaa gca aat atc 1107
 Ile His Phe Cys Leu Gly Ala Pro Leu Ala Arg Leu Glu Ala Asn Ile
 35 350 355 360

365 370 375

40 gcg tta acg tct ttg att tct gct ttt cct cat atg gag tgc gtc agt 1155
 Ala Leu Thr Ser Leu Ile Ser Ala Phe Pro His Met Glu Cys Val Ser
 45 365 370 375

45 atc act ccg att gaa aac agt gtg ata tac gga tta aag agc ttc cgt 1203
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 50 380 385 390

55 gtg aaa atg taaggatcc 1221
 Val Lys Met

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<211> 1221

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<213> *Bacillus subtilis*

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<221> CDS

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<221> (25)..(1212)

<400> 44

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Met Asn Val Leu Asn Arg Arg Gln Ala

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ttg ccg cga gca ctg ctc aat ggg aaa aac aaa cag gat gcg tat cat 99

Leu Pro Arg Ala Leu Leu Asn Gly Lys Asn Lys Gln Asp Ala Tyr His

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ccg ttt cca tgg tat gaa tcg atg aga aag gat gcg cct gtt tcc ttt 147

Pro Phe Pro Trp Tyr Glu Ser Met Arg Lys Asp Ala Pro Val Ser Phe

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gat gaa gaa aac caa gtg tgg agc gtt ttt ctt tat gat gat gtc aaa 195

Asp Glu Glu Asn Gln Val Trp Ser Val Phe Leu Tyr Asp Asp Val Lys

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aaa gtt gtt ggg gat aaa gag ttg ttt tcc aat gtc atg ccc cag cag 243

Lys Val Val Gly Asp Lys Glu Leu Phe Ser Ser Cys Met Pro Gln Gln
 5 60 65 70

aca agc tct att gga aat tcc atc att agc atg gac ccg ccg aag cat 291
 10 Thr Ser Ser Ile Gly Asn Ser Ile Ile Ser Met Asp Pro Pro Lys His
 75 80 85

aca aaa atc cgt tca gtc gtg aac aaa gcc ttt act ccg cgc gcg atg 339
 15 Thr Lys Ile Arg Ser Val Val Asn Lys Ala Phe Thr Pro Arg Ala Met
 20 90 95 100 105

aag caa tgg gaa ccg aga att caa gaa atc aca gat gaa ctg att caa 387
 25 Lys Gln Trp Glu Pro Arg Ile Gln Glu Ile Thr Asp Glu Leu Ile Gln
 110 115 120

aza ttt cag ggg cgc agt gag ttt gac ctt gtt cac gat tat tca tac 435
 30 Lys Phe Gln Gly Arg Ser Glu Phe Asp Leu Val His Asp Tyr Ser Tyr
 35 125 130 135

ccg ctt ccg gtt att gtg ata tct gag ctg ctg gga gtg cct tca gcg 483
 40 Pro Leu Pro Val Ile Val Ile Ser Glu Leu Leu Gly Val Pro Ser Ala
 140 145 150

cat atg gaa cag ttt aaa gca tgg tct gat ctt ctg gtc agt aca ccg 531
 45 His Met Glu Gln Phe Lys Ala Trp Ser Asp Leu Leu Val Ser Thr Pro
 50 155 160 165

aag gat aza agt gaa gaa gct gaa aaa gcc ttt ttg gaa gaa cga gat 579
 55 Lys Asp Lys Ser Glu Glu Ala Glu Lys Ala Phe Leu Glu Glu Arg Asp

170	175	180	185
5			
aag tgt gag gaa gaa ctg gcc gcg ttt ttt gcc ggc atc ata gaa gaa			627
Lys Cys Glu Glu Glu Leu Ala Ala Phe Phe Ala Gly Ile Ile Glu Glu			
190	195	200	
10			
aag cga aac aaa ccg gaa cag gat att att tct att tta gtg gaa gcg			675
Lys Arg Asn Lys Pro Glu Gln Asp Ile Ile Ser Ile Leu Val Glu Ala			
205	210	215	
15			
gaa gaa aca ggc gag aag ctg tcc ggt gaa gag ctg att ccg ttg tgc			723
Glu Glu Thr Gly Glu Lys Leu Ser Gly Glu Glu Leu Ile Pro Leu Cys			
220	225	230	
20			
acg ctg ctg ctg gtg gcc gga aat gaa acc act aca aac ctg att tca			771
Thr Leu Leu Leu Val Ala Gly Asn Glu Thr Thr Asn Leu Ile Ser			
235	240	245	
25			
aat gcg atg ttc agc ata tta gaa acg cca ggc gtt tac gag gaa ctg			819
Asn Ala Met Phe Ser Ile Leu Glu Thr Pro Gly Val Tyr Glu Glu Leu			
250	255	260	265
40			
cgc agc cat cct gaa ctg atg ccc cag gca gtg gag gaa gcc ttg cgt			867
Arg Ser His Pro Glu Leu Met Pro Gln Ala Val Glu Glu Ala Leu Arg			
270	275	280	
45			
ttc aga gcg ccg gcc ccg gtt ttg agg cgc att gcc aag cgg gat acg			915
Phe Arg Ala Pro Ala Pro Val Leu Arg Arg Ile Ala Lys Arg Asp Thr			
285	290	295	
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5 <211> 396

<212> PRT

10 <213> *Bacillus subtilis*

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20 25 3025 Met Arg Lys Asp Ala Pro Val Ser Phe Asp Glu Glu Asn Gln Val Trp
35 40 4530 Ser Val Phe Leu Tyr Asp Asp Val Lys Lys Val Val Gly Asp Lys Glu
35 50 55 6040 Leu Phe Ser Ser Cys Met Pro Gln Gln Thr Ser Ser Ile Gly Asn Ser
65 70 75 8045 Ile Ile Ser Met Asp Pro Pro Lys His Thr Lys Ile Arg Ser Val Val
85 90 9550 Asn Lys Ala Phe Thr Pro Arg Ala Met Lys Gln Trp Glu Pro Arg Ile
100 105 110

5 Gln Glu Ile Thr Asp Glu Leu Ile Gln Lys Phe Gln Gly Arg Ser Glu
115 120 125

10 Phe Asp Leu Val His Asp Tyr Ser Tyr Pro Leu Pro Val Val Ile
130 135 140

15 Ser Glu Leu Leu Gly Val Pro Ser Ala His Met Glu Gln Phe Lys Ala
145 150 155 160

20 Trp Ser Asp Leu Leu Val Ser Thr Pro Lys Asp Lys Ser Glu Glu Ala
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25 Glu Lys Ala Phe Leu Glu Glu Arg Asp Lys Cys Glu Glu Leu Ala
180 185 190

30 Ala Phe Phe Ala Gly Ile Ile Glu Glu Lys Arg Asn Lys Pro Glu Gln
195 200 205

35 Asp Ile Ile Ser Ile Leu Val Glu Ala Glu Glu Thr Gly Glu Lys Leu
210 215 220

40 Ser Gly Glu Glu Leu Ile Pro Leu Cys Thr Leu Leu Leu Val Ala Gly
225 230 235 240

45 Asn Glu Thr Thr Asn Leu Ile Ser Asn Ala Met Phe Ser Ile Leu
245 250 255

55 Glu Thr Pro Gly Val Tyr Glu Glu Leu Arg Ser His Pro Glu Leu Met
260 265 270

5 Pro Gln Ala Val Glu Glu Ala Leu Arg Phe Arg Ala Pro Ala Pro Val
 275 280 285

10 Leu Arg Arg Ile Ala Lys Arg Asp Thr Glu Ile Gly Gly His Leu Ile
 290 295 300

15 Lys Glu Gly Asp Thr Val Leu Ala Phe Val Ala Ser Ala Asn Arg Asp
 305 310 315 320

20 Glu Ala Lys Phe Asp Arg Pro His Met Phe Asp Ile Arg Arg His Pro
 325 330 335

25 Asn Pro His Ile Ala Phe Gly His Gly Ile His Phe Cys Leu Gly Ala
 340 345 350

30 Pro Leu Ala Arg Leu Glu Ala Asn Ile Ala Leu Thr Ser Leu Ile Ser
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35 Ala Phe Pro His Met Glu Cys Val Ser Ile Thr Pro Ile Glu Asn Ser
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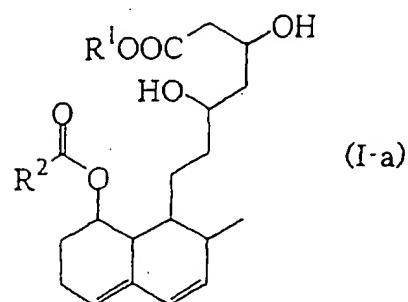
40 Val Ile Tyr Gly Leu Lys Ser Phe Arg Val Lys Met
 385 390 395

50 Claims

55 1. A protein which is derived from a microorganism belonging to the genus *Bacillus*, and has an activity of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b),
 wherein the compound (I-a) is a compound represented by the formula (I-a):

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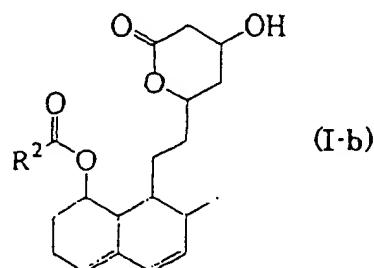
15

wherein R¹ represents a hydrogen atom, a substituted or unsubstituted alkyl, or an alkali metal, and R² represents a substituted or unsubstituted alkyl, or a substituted or unsubstituted aryl;
 the compound (I-b) is a lactone form of compound (I-a) and is represented by the formula (I-b):

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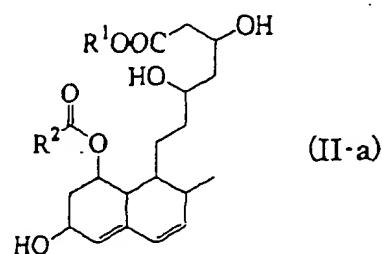


35

wherein R² has the same definition as the above;
 the compound (II-a) is a compound represented by the formula (II-a):

40

45



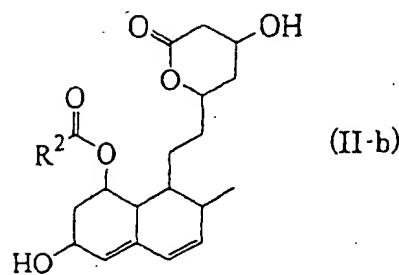
50

55

wherein R¹ and R² have the same definitions as the above; and
 the compound (II-b) is a lactone form of compound (II-a) and is represented by the formula (II-b):

5

10



wherein R² has the same definition as the above.

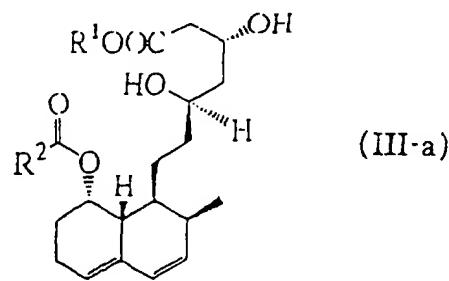
15

2. A protein which is derived from a microorganism belonging to the genus *Bacillus*, and has an activity of producing compound (IV-a) or compound (IV-b) from compound (III-a) or compound (III-b), wherein the compound (III-a) is a compound represented by the formula (III-a):

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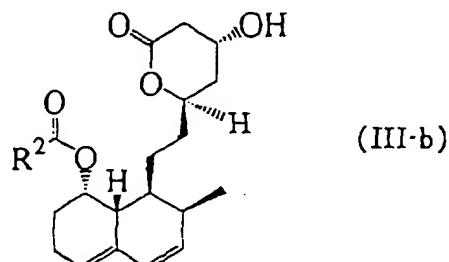
30



wherein R¹ represents a hydrogen atom, a substituted or unsubstituted alkyl, or an alkali metal, and R² represents a substituted or unsubstituted alkyl, or a substituted or unsubstituted aryl:
the compound (III-b) is a lactone form of compound (III-a) and is represented by the formula (III-b):

40

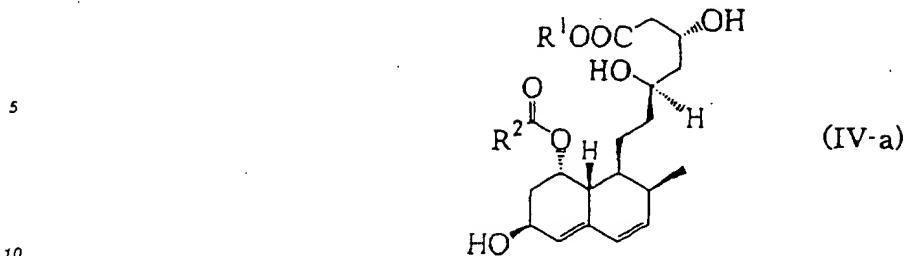
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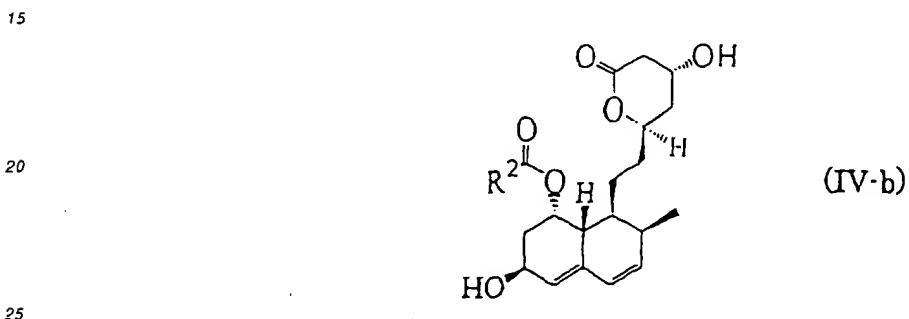
50

wherein R² has the same definition as the above;
the compound (IV-a) is a compound represented by the formula (IV-a):

55



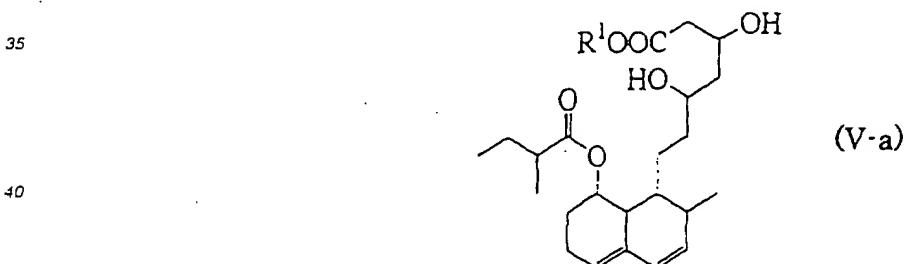
wherein R¹ and R² have the same definitions as the above; and
the compound (IV-b) is a lactone form of compound (IV-a) and is represented by the formula (IV-b):



wherein R² has the same definition as the above.

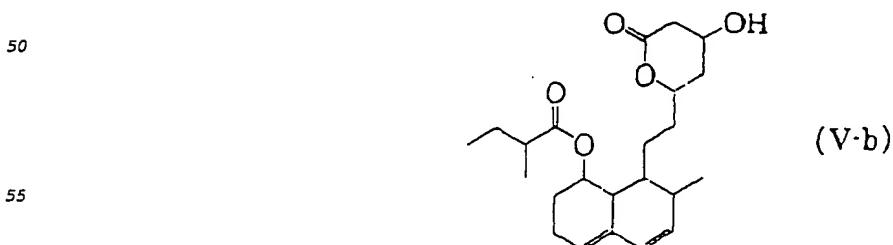
30

3. A protein which is derived from a microorganism belonging to the genus *Bacillus*, and has an activity of producing compound (VI-a) or compound (VI-b) from compound (V-a) or compound (V-b),
wherein the compound (V-a) is a compound represented by the formula (V-a):



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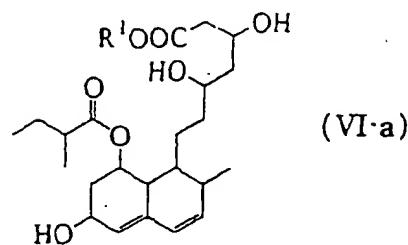
wherein R¹ represents a hydrogen atom, a substituted or unsubstituted alkyl, or an alkali metal;
the compound (V-b) is a lactone form of compound (V-a) and is represented by the formula (V-b):



the compound (VI-a) is a compound represented by the formula (VI-a):

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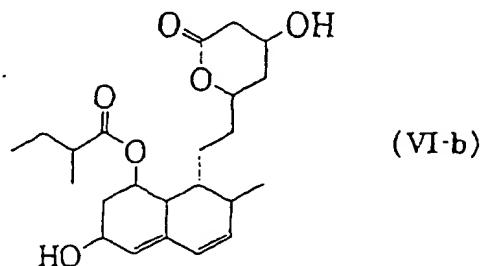
15

wherein R^1 has the same definition as the above; and

the compound (VI-b) is a lactone form of the compound (VI-a) and is represented by the formula (VI-b):

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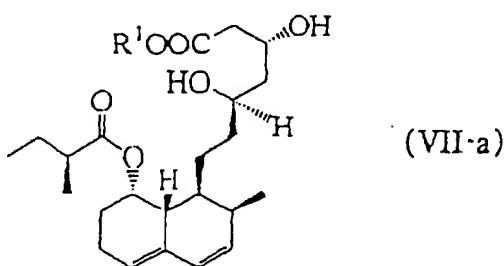


30

4. A protein which is derived from a microorganism belonging to the genus *Bacillus*, and has an activity of producing compound (VIII-a) or compound (VIII-b) from compound (VII-a) or compound (VII-b),
wherein the compound (VII-a) is a compound represented by the formula (VII-a):

35

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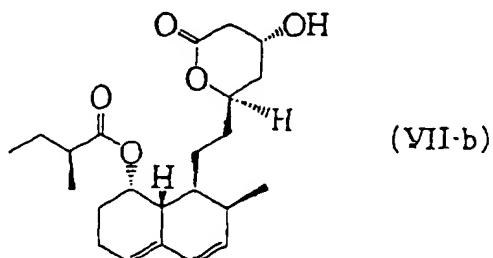


45

wherein R^1 represents a hydrogen atom, a substituted or unsubstituted alkyl, or an alkali metal;
the compound (VII-b) is a lactone form of compound (VII-a) and is represented by the formula (VII-b):

50

55

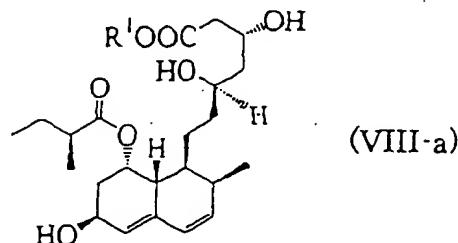


the compound (VIII-a) is a compound represented by the formula (VIII-a):

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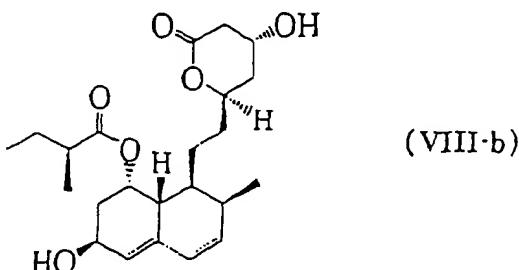
50

55

60

wherein R¹ has the same definition as the above; and

the compound (VIII-b) is a lactone form of compound (VIII-a) and is represented by the formula (VIII-b):



5. The protein according to any one of claims 1 to 4, wherein the microorganism belonging to the genus *Bacillus* is a microorganism selected from *B. subtilis*, *B. megaterium*, *B. laterosporus*, *B. sphaericus*, *B. pumilus*, *B. stearothermophilus*, *B. cereus*, *B. badius*, *B. brevis*, *B. alvei*, *B. circulans* and *B. macerans*.
6. The protein according to any one of claim 1 to 5, wherein the microorganism belonging to the genus *Bacillus* is a microorganism selected from *B. subtilis* ATCC6051, *B. megaterium* ATCC10778, *B. megaterium* ATCC11562, *B. megaterium* ATCC13402, *B. megaterium* ATCC15177, *B. megaterium* ATCC15450, *B. megaterium* ATCC19213, *B. megaterium* IAM1032, *B. laterosporus* ATCC4517, *B. pumilus* FERM BP-2064, *B. badius* ATCC14574, *B. brevis* NRRL B-8029, *B. alvei* ATCC6344, *B. circulans* NTCT-2610, and *B. macerans* NCIMB-9368.
7. The protein according to any one of claims 1 to 5, wherein the microorganism belonging to the genus *Bacillus* is a microorganism selected from *Bacillus* sp. FERM BP-6029 or *Bacillus* sp. FERM BP-6030.
8. A protein having the amino acid sequence shown by SEQ ID NO: 1.
9. A protein which has an amino acid sequence comprising deletion, substitution or addition of one or more amino acids in the amino acid sequence shown by SEQ ID NO: 1, and has an activity of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b).
10. The protein according to claim 9, wherein the protein has the amino acid sequence shown by SEQ ID NO: 42 or 45.
11. The protein according to claim 9, wherein the compound (I-a) is compound (III-a), the compound (I-b) is compound (III-b), the compound (II-a) is compound (IV-a), and the compound (II-b) is compound (IV-b).
12. The protein according to claim 9, wherein the compound (I-a) is compound (V-a), the compound (I-b) is compound (V-b), the compound (II-a) is compound (VI-a), and the compound (II-b) is compound (VI-b).
13. The protein according to claim 9, wherein the compound (I-a) is compound (VII-a), the compound (I-b) is compound

(VII-b), the compound (II-a) is compound (VIII-a), and the compound (II-b) is compound (VIII-b).

14. An isolated DNA having the nucleotide sequence shown by SEQ ID NO: 2.
- 5 15. An isolated DNA which hybridizes with the DNA according to claim 14 under stringent conditions, and encodes a protein having an activity of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b).
- 10 16. The DNA according to claim 15, wherein the DNA has a nucleotide sequence selected from the group consisting of the nucleotide sequences shown by SEQ ID NOS: 41, 43 and 44.
17. An isolated DNA encoding the protein according to any one of claims 1 to 12.
- 15 18. The DNA according to claim 15, wherein the compound (I-a) is compound (III-a), the compound (I-b) is compound (III-b), the compound (II-a) is compound (IV-a), and the compound (II-b) is compound (IV-b).
19. The DNA according to claim 15, wherein the compound (I-a) is compound (V-a), the compound (I-b) is compound (V-b), the compound (II-a) is compound (VI-a), and the compound (II-b) is compound (VI-b).
- 20 21. The DNA according to claim 15, wherein the compound (I-a) is compound (VII-a), the compound (I-b) is compound (VII-b), the compound (II-a) is compound (VIII-a), and the compound (II-b) is compound (VIII-b).
- 25 22. A recombinant DNA vector comprising the DNA according to any one of claims 14 to 20.
23. The transformant obtained by introducing the recombinant DNA vector according to claim 21 into a host cell.
- 30 24. The transformant according to claim 22, wherein the transformant belongs to a microorganism selected from the genera *Escherichia*, *Bacillus*, *Corynebacterium*, and *Streptomyces*.
- 35 25. The transformant according to claim 22 or 23, wherein the transformant belongs to microorganism selected from *Escherichia coli*, *Bacillus subtilis*, *Bacillus megaterium*, *Corynebacterium glutamicum*, *Corynebacterium ammoniagenes*, *Corynebacterium callunae* and *Streptomyces lividans*.
- 35 26. A process for producing compound (II-a) or compound (II-b), wherein the transformant according to any one of claims 22 to 24, a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:
 - allowing compound (I-a) or compound (I-b) to exist in an aqueous medium;
 - allowing compound (II-a) or compound (II-b) to be produced and accumulated in said aqueous medium; and
 - collecting compound (II-a) or compound (II-b) from said aqueous medium.
- 40 26. A process for producing compound (IV-a) or compound (IV-b), wherein the transformant according to any one of claims 22 to 24, a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:
 - allowing compound (III-a) or compound (III-b) to exist in an aqueous medium;
 - allowing compound (IV-a) or compound (IV-b) to be produced and accumulated in said aqueous medium; and
 - collecting compound (IV-a) or compound (IV-b) from said aqueous medium.
- 45 27. A process for producing compound (VI-a) or compound (VI-b), wherein the transformant according to any one of claims 22 to 24, a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:
 - allowing compound (V-a) or compound (V-b) to exist in an aqueous medium;
 - allowing compound (VI-a) or compound (VI-b) to be produced and accumulated in said aqueous medium; and
 - collecting compound (VI-a) or compound (VI-b) from said aqueous medium.
- 50 28. A process for producing compound (VIII-a) or compound (VIII-b), wherein the transformant according to any one of claims 22 to 24, a culture of the transformant, or a treated product of the culture is used as an enzyme source.

and the process comprises:

allowing compound (VII-a) or compound (VII-b) to exist in an aqueous medium;
allowing compound (VIII-a) or compound (VIII-b) to be produced and accumulated in said aqueous medium;
5 and
collecting compound (VIII-a) or compound (VIII-b) from said aqueous medium.

29. The process according to claim 25, wherein the compound (II-b) is the compound (II-b) obtained by forming a lacton from compound (II-a).
- 10 30. The process according to claim 25, wherein the compound (II-a) is the compound (II-a) obtained by opening the lactone ring of compound (II-b).
- 15 31. The process according to claim 26, wherein the compound (IV-b) is the compound (IV-b) obtained by forming a lacton from compound (IV-a).
32. The process according to claim 26, wherein the compound (IV-a) is the compound (IV-a) obtained by opening the lactone ring of compound (IV-b).
- 20 33. The process according to claim 27, wherein the compound (VI-b) is the compound (VI-b) obtained by forming a lacton from compound (VI-a).
34. The process according to claim 27, wherein the compound (VI-a) is the compound (VI-a) obtained by opening the lactone ring of compound (VI-b).
- 25 35. The process according to claim 28, wherein the compound (VIII-b) is the compound (VIII-b) obtained by forming a lacton from compound (VIII-a).
- 30 36. The process according to claim 28, wherein the compound (VIII-a) is the compound (VIII-a) obtained by opening the lactone ring of compound (VIII-b).
37. The process according to any one of claims 25 to 28, wherein the treated product of the culture of the transformant is a treated product selected from cultured cells; treated products such as dried cells, freeze-dried cells, cells treated with a surfactant, cells treated with an enzyme, cells treated by ultrasonication, cells treated by mechanical milling, cells treated by solvent; a protein fraction of a cell; and an immobilized products of cells or treated cells.
- 35 38. A process for producing a protein, which comprises culturing the transformant according to any one of claims 22 to 24 in a medium; producing and accumulating the protein according to any one of claims 1 to 12 in the culture; and collecting said protein from said culture.
- 40 39. An oligonucleotide corresponding to a sequence consisting of 5 to 60 continuous nucleotides in a nucleotide sequence selected from the group consisting of the nucleotide sequences shown by SEQ ID NOS: 2, 41, 43 and 44; or an oligonucleotide corresponding to a complementary sequence to said oligonucleotide.

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/00472

A. CLASSIFICATION OF SUBJECT MATTER
 Int.Cl' C12N9/02, 1/21, 15/53, C12P7/62, 17/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 Int.Cl' C12N9/00-9/02, 15/52-15/53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
 GenBank/EMBL/DBDBJ/GeneSeq, SwissProt/PIR/GeneSeq,
 BIOSIS (DIALOG), WPI (DIALOG)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Nature, Volume 390, issued November 20, 1997, F. Kunst et al., "The complete genome sequence of the Gram-positive bacterium <i>Bacillus subtilis</i> ", pages 249-256 & Database GenBank Accession No. B69851	1-24,39
X	Microbiology, Volume 144, Part 4, issued April 1998, Caric Rivolta et al., "A 35 · 7 kb DNA fragment from the <i>Bacillus subtilis</i> chromosome containing a putative 12 · 3 kb operon involved in hexuronate catabolism and a perfectly symmetrical hypothetical catabolite-responsive element", pages 877-884 & Database GenBank Accession No. AF015825	1-24,39
A	EP, 649907, A1 (BRISTOL-MYERS SQUIBB COMPANY), 26 April, 1995 (26.04.95) & HU, 217104, B & AU, 9475972, A & FI, 9404926, A & CA, 2134025, A & JP, 7-184670, A & CN, 1106067, A & IL, 111084, A & SG, 47504, A1	1-39

Further documents are listed in the continuation of Box C. See patent family annex.

• Special categories of cited documents:	
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"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed
"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search 27 April, 2000 (27.04.00)	Date of mailing of the international search report 16 May, 2000 (16.05.00)
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INTERNATIONAL SEARCH REPORT		International application No. PCT/JP00/00472
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP, 64-2585, A (SANKYO COMPANY, LIMITED), 06 January, 1989 (06.01.89) (Family: none)	1-39
A	Cardiology, Volume 77, Supplement 4, issued October 1990, Alfred W. Alberts, "Lovastatin and Simvastatin - Inhibitors of HMG CoA Reductase and Cholesterol Biosynthesis", pages 14-21	1-39
A	The Journal of Antibiotics, Volume 29, Number 12, issued December 1976, Akira Endo et al., "ML-236A, ML-236B, AND ML-236C, NEW INHIBITORS OF CHOLESTEROGENESIS PRODUCED BY PENICILLIUM CITRINUM", pages 1346-1348	1-39